(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
26 September 2002 (26.09.2002)

PCT

(10) International Publication Number
WO 02/074920 A2

(51) International Patent Classification[7]: C12N

(21) International Application Number: PCT/US02/08033

(22) International Filing Date: 18 March 2002 (18.03.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/276,854 16 March 2001 (16.03.2001) US

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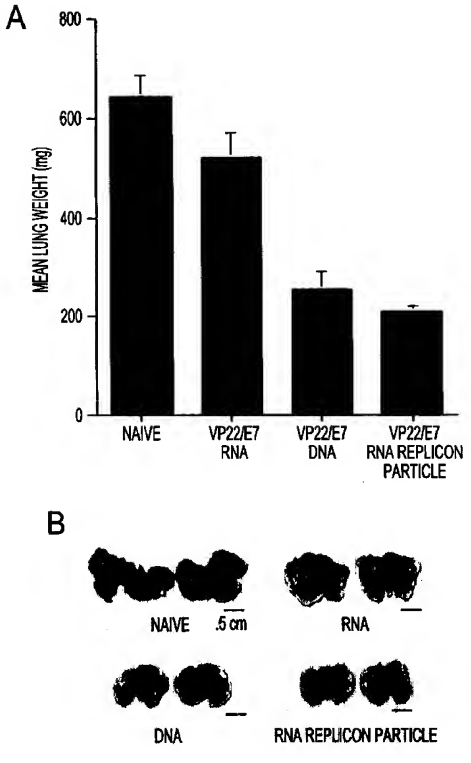
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent

[Continued on next page]

(54) Title: MOLECULAR VACCINE LINKING ANTIGEN WITH AN IMMUNOGENICITY-POTENTIATING POLYPEPTIDE DELIVERED AS REPLICATION DEFECTIVE ALPHAVIRUS REPLICONS FROM STABLE PACKAGING CELLS



(57) Abstract: Superior molecular vaccines comprise nucleic acids in the form of PCL-generated replication-defective alphavirus replicons, preferably Sindbis virus, that encode a fusion polypeptide that includes an antigenic peptide or polypeptide against which an immune response is desired. Fused to the antigenic peptide is at least a second polypeptide that is an immunogenicity-potentiating olypeptide acting by any of a number of mechanisms to promote immunogenicity of the antigen. Examples include intercellular spreading proteins, in particular a herpes virus protein VP22 or a homologue or functional derivative thereof. Other examples are proteins that stimulate MHC class I processing of the antigen, target the antigen to APCs promote development and growth of immature DCs or stimulate DC antigen presenting activity. The nucleic acid can encode any antigenic epitope of interest, preferably an epitope that is processed and presented by MHC class I proteins. Antigens of pathogenic organisms and cells such as tumor cells are preferred. Vaccines comprising HPV-16 E7 oncoprotein are exemplified. Also disclosed are methods of using the vaccines to induce heightened T cell mediated immunity, in particular by cytotoxic T lymphocytes, leading to protection from or treatment of a tumor.

WO 02/074920 A2

(BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declaration under Rule 4.17:

— *of inventorship (Rule 4.17(iv)) for US only*

Published:

— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

MOLECULAR VACCINE LINKING ANTIGEN WITH AN IMMUNOGENICITY-POTENTIATING POLYPEPTIDE DELIVERED AS REPLICATION DEFECTIVE ALPHAVIRUS REPLICONS FROM STABLE PACKAGING CELLS

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention in the fields of molecular biology, immunology and medicine relates to a PCL-generated replication-defective alphavirus replicons as vectors for chimeric nucleic acid vaccines encoding fusion proteins. These vectors are used as vaccines to enhance immune responses, primarily cytotoxic T lymphocyte (CTL) responses to specific antigens such as tumor or viral antigens. The fusion protein comprises an antigenic polypeptide fused to an immunogenicity-potentiating polypeptide that promotes intercellular transport of the antigen, processing via the MHC class I pathway, stimulation of dendritic cell development or function, and the like.

Description of the Background Art

Naked DNA vaccines have emerged as attractive approaches for vaccine development (Hoffman, SL *et al.*, 1995, *Ann N Y Acad Sci 772:88-94;* Donnelly, JJ *et al.*, 1997, *Annu Rev Immunol 15:617-648*; Gurunathan, S *et al.*, 2000, *Annu Rev Immunol 18:927-74*). Intradermal administration of DNA vaccines via gene gun represents a convenient way of delivering DNA vaccines into professional antigen presenting cells (APCs) *in vivo*. Professional APCs are a superior candidate for mediating presentation of an antigen encoded by such a DNA vaccine to T lymphocytes of the immune system. The "gene gun" strategy provides efficient delivery of DNA into epidermal bone marrow-derived APCs termed Langerhans cells, which move to draining lymph nodes where they enter the lymphatic system. The present inventors and their colleagues have successfully used this system of DNA delivery to test various intracellular targeting strategies (Chen *et al.*, 2000, Cancer Res. 60:1035–1042; Ji *et al.*, 1999, *Human Gene Therapy 10:2727-2740*); co-pending, commonly assigned U.S. patent applications USSN 09/421,608; 09/501,097, 09/693,450??? and 60/281,003???).

Recently, self-replicating RNA vaccines (RNA replicons) have also emerged as an important strategy to enhance the potency of nucleic acid vaccines for cancer immunotherapy (for review, see Leitner, W *et al.*, 1999, *Vaccine 18*:765–777. RNA replicon vaccines may be derived from alphavirus vectors, such as Sindbis virus (Hariharan, JM *et al.*, 1998, *J. Virol.*

72:950–958), Semliki Forest virus (Berglund, P *et al.*, 1997, *AIDS Res. Hum. Retrovir. 13*:1487–1495; Berglund, P. *et al.*, 1998, *Nat. Biotech. 16*:562–565) or Venezuelan equine encephalitis virus (Pushko, P *et al.*, 1997, *Virology 239*:389–401) vectors. These vaccines are self-replicating and self-limiting and may be administered as either RNA or DNA, which is then transcribed into RNA replicons in transfected cells or *in vivo* (Berglund *et al.*, *supra*; Leitner, WW *et al.*, 2000, *Cancer Res. 60*:51–55). Self-replicating RNA eventually causes lysis of transfected cells (Ying, H *et al.*, 1999, *Nat. Med. 5*:823–827). These vectors do not raise the concern about integration into the host genome associated with naked DNA vectors. This is particularly important for development of vaccines that target potentially oncogenic proteins such as the human papillomavirus (HPV) E6 and E7 proteins. One limitation on the potency of RNA replicon vaccines is their inability to spread *in vivo*. The present inventors conceived a strategy that facilitates the spread of antigen to enhance significantly the potency of RNA replicon vaccines.

Alphavirus vectors, such as Sindbis virus (Hariharan *et al.*, 1998; Xiong *et al.*, 1989. *Science 243*:1188-1191) and Semliki Forest virus (Berglund *et al.*, 1997, AIDS Res Hum Retroviruses *13*:1487-1495; Daemen *et al.*, 2000, *Gene Ther. 7*:1859-1866), have become an important strategy for the development of vaccines and gene therapy applications because of their high levels of RNA replication and gene expression in cells, their ability to infect a variety of diverse cell types, and the relative ease of manipulating cDNA clones for transcription of vectors and infectious viral RNA (for review, see (Dubensky *et al.*, In: *Gene Therapy: Therapeutic Mechanisms and Strategies*. Templeton, NS *et al.*, eds, pp.109-129, Marcel Dekker Inc: New York, 2000.; Frolov *et al.*, 1996, Proc Natl Acad Sci U S A. *93*:11371-11377; Garoff & Li, 1998, Curr Opin Biotechnol. **9**, 464-469; Huang, HV, 1996, Curr Opin Biotechnol. **7**, 531-535; Schlesinger & Dubensky, 1999, Curr Opin Biotechnol. ***10***:434-439 ; Strauss & Strauss, 1994, Microbiol Rev. *58*:491-562)). The general strategy for construction of alphavirus-based expression vectors has been to substitute viral structural protein genes with a heterologous gene, while preserving transcriptional control via the highly active subgenomic RNA promoter (Frolov *et al.*, *supra*; Huang, *supra*; Xiong *et al.*, *supra*). These vectors are self-replicating in cells and may be administered as either RNA, DNA, or infectious propagation-incompetent alphavirus particles. Since alphavirus vectors eventually trigger apoptosis of transfected cells (Ying *et al.*, 1999, Nat Med. *5*:823-827), they do not raise the concern associated with DNA integration into

the host genome. This is particularly important for vaccine development approaches targeting proteins that are potentially oncogenic, such as the HPV E6 and E7 proteins.

Unlike DNA- or RNA-based nucleic acid vaccines, infectious alphavirus replicon particles provide a highly efficient method of introducing heterologous genes into target cells and the opportunity to generate vaccines at a large scale. However, significant concerns have been raised about potential contamination with replication-competent virus. Recently, an alphavirus replicon packaging cell line (PCL) was developed (Polo, JM *et al.*, 1999, Proc Natl Acad Sci U S A. *96*:4598-4603) to produce alphavirus replicon particle stocks, including Sindbis virus and Semliki Forest virus-derived vectors, free of detectable contaminating replication-competent virus. In this PCL (987dlsplit #24), genes encoding the capsid and envelope glycoproteins were separated into distinct cassettes, resulting in undetectable levels of contaminating replication-competent virus while maintaining relatively high levels of viral particle production (approximately 10^7 infectious units/ml). The availability of such a PCL allows for large-scale vector production that may be useful in vaccine applications.

HSP70: A Protein that Promotes Antigen Processing via the MHC Class I Pathway

The present inventors and their colleagues (Chen (2000) *Canc Resh 60*:1035-1042) demonstrated that linkage of human papillomavirus type 16 (HPV-16) E7 antigen to *Mycobacterium tuberculosis* heat shock protein 70 (HSP70) led to enhancement of DNA vaccine potency. Other studies have demonstrated that immunization with heat shock protein (HSP) complexes isolated from tumor or virus-infected cells are able to induce potent anti-tumor (Janetzki (1998) J. Immunother. 21:269-276) or antiviral immunity (Heikema (1997) Immunol. Lett. 57:69-74). Some HSP-based protein vaccines involved fusing antigens to HSPs (Suzue (1996) J. Immunol. 156:873-879) wherein, HSP70 fusion protein elicited humoral and cellular immune responses to an HIV-1 protein. While these investigations have made HSPs more attractive for use in immunotherapy, there have been no reports or suggestions of making HSP-linked molecular vaccine using replication defective alphavirus vectors as described herein.

The centrosome, also called the microtubule organizing center (MTOC), is a perinuclear organelle which contains a high density of proteasomes (Anton, LC *et al.*, 1999, J Cell Biol. *146:* 113-24; Wigley, WC, 1999, J Cell Biol. *145:*481-90; Fabunmi, RP *et al.*, J Biol Chem. *275:*409-13, 2000). Several proteins, notably γ-tubulin and β-tubulin, are localized and

concentrated at the centrosome. The centrosome has been implicated as an important intracellular compartment for proteasomal degradation of certain antigens (Anton, *supra*).

Endoplasmic Reticulum Chaperone Polypeptides

Calreticulin (CRT), an abundant 46 kilodalton (kDa) protein located in the lumen of the cell's endoplasmic reticulum (ER), displays lectin activity and participates in the folding and assembly of nascent glycoproteins. See, *e.g.*,, Nash (1994) *Mol. Cell. Biochem. 135*:71-78; Hebert (1997) *J. Cell Biol. 139*:613-623; Vassilakos (1998) *Biochemistry 37*:3480-3490; Spiro (1996) *J. Biol. Chem.* 271:11588-11594. CRT associates with peptides transported into the ER by transporters that are associated with antigen processing, such as TAP-1 and TAP-2 (Spee (1997) *Eur. J. Immunol. 27*:2441-2449). CRT also forms complexes with peptides *in vitro*. Upon adminsitration to mice, these complexes, elicited peptide-specific CD8+ T cell responses (Basu (1999) *J. Exp. Med. 189*:797-802; Nair (1999) *J. Immunol. 162*:6426-6432). CRT purified from murine tumors elicited immunity specific for the tumor from which the CRT was taken, but not for an antigenically distinct tumor (Basu, *supra*). By pulsing mouse dendritic cells (DCs) *in vitro* with a CRT-peptide complex, the peptide was re-presented by MHC class I molecules on the DCs to stimulate a peptide-specific CTL response(Nair, *supra*).

CRT also has anti-angiogenic effects. CRT and a fragment comprising amino acid residues 1-180, which has been called "vasostatin," are endothelial cell inhibitors that can suppress tumor growth (Pike (1999) Blood. 94:2461-2468). Tumor growth and metastasis depend on the existence of an adequate blood supply. As tumors grow larger, adequate blood supply to the tumor tissue is often ensured by new vessel formation, a process termed angiogenesis. (Folkman (1982) *Ann. NY Acad. Sci. 401*:212-27; Hanahan (1996) *Cell 86*:353-364). Therapeutic agents that target and damage tumor vasculature can prevent or delay tumor growth and even promote regression or dormancy.

Viral Polypeptides that Promote Intercellular Transport and Spread

One limitation of DNA vaccines is their potency, since they do not have the intrinsic ability to amplify and spread *in vivo* as some replicating viral vaccine vectors do. The present inventors conceived a strategy that facilitates the spread of antigen may significantly enhance the potency of naked DNA vaccines.

VP22, a herpes simplex virus (HSV-1) protein has demonstrated the remarkable property of intercellular transport and is capable of distributing protein to many surrounding cells(4) (U.S. Patent 6,017,735, O'Hare & Elliott, 25 Jan 2000). For example, VP22 has been linked to p53 (Phelan, A. *et al.*, 1998, *Nat Biotechnol 16:440-3*) or thymidine kinase (Dilber, MS *et al.*, 1999, *Gene Ther 6:12-21*), facilitating the spread of linked proteins to surrounding cells *in vitro* and the treatment of model tumors. Marek's disease virus type 1 (MDV-1) UL49 shares homology with HSV-1 VP22 (Koptidesova *et al.*, 1995, *Arch Virol. 140*:355-362) and has been shown to be capable of intercellular transport after exogenous application (Dorange *et al.*, 2000, *J Gen Virol. 81 Pt 9*:2219-2230).

Polypeptide Stimulators of Growth, Differentiation or Activation of Antigen Presenting Cells

A molecule that stimulates growth of DC precursors and can help in generating large numbers of DCs *in vivo* is Flt3-ligand ("FL") (Maraskovsky, E *et al., J Exp Med 184*: 1953-62, 1996, Shurin, MR *et al., Cell Lmmunol. 179:* 174-84, 1997). FL has emerged as an important molecule in the development of tumor vaccines that augment numbers and action of DCs *in vivo*. Flt3, a murine tyrosine kinase receptor, first described in 1991 (Rosnet, O *et al., Oncogene.* 6: 1641-50, 1991), was found to be a member of the type III receptor kinase family which includes - kit and c-fms (for review, see (Lyman, SD *Curr Opin Hematol.* 5:192-6, 1998). In hematopoietic tissues, the Flt3expression is restricted to the CD34+ progenitor population. Flt3 has been used to identify and subsequently clone the corresponding ligand, Flt3-ligand or "FL" (Lyman, SD *et al., Cell. 75:* 1157-67, 1993; Hannum, C *et al., Nature. 368:* 643-8, 1994).

The predominant form of FL is synthesized as a transmembrane protein from which the soluble form is believed to be generated by proteolytic cleavage. The soluble form of FL (the extracellular domain or "ECD") is functionally similar to intact FL (Lyman, SD *et al., Cell. 75*: 1157-67, 1993). These proteins function by binding to and activating unique tyrosine kinase receptors. Expression of the Flt3 receptor is primarily restricted, among hematopoietic cells, to the most primitive progenitor cells, including DC precursors. The soluble ECD of FL induced strong anti-tumor effects against several murine model tumors including fibrosarcoma (Lynch, DH *et al., Nat Med. 3*: 625-31, 1997), breast cancer (Chen, K et al *Cancer Res. 57*: 3511-6, 1997; Braun, SE *et al., Hum Gene Ther. 10:* 2141-51, 1999), liver cancer (Peron, JM *et al., J*

Immunol. 161: 6164-70, 1998), lung cancer (Chakravarty, PK *et al., Cancer Res. 59*: 6028-32, 1999), melanoma and lymphoma (Esche, C *et al., Cancer Res. 58*: 380-3, 1998).

SUMMARY OF THE INVENTION

The potency of naked DNA molecular vaccines is limited by their inability to amplify and spread *in vivo*. Inclusion of nucleic acid sequences that encode polypeptides that modify the way the antigen encoded by molecular vaccine is "received" or "handled" by the immune system serve as a basis for enhancing vaccine potency. Polypeptides that have such modes of action are termed herein "*i*mmunogenicity-potentiating (or -promoting) polypeptide" or "IPP" to reflect this general property, even though these IPP's may act by any of a number of cellular and molecular mechanisms that may or may not share common steps. IPP's may be produced as fusion or chimeric polypeptides with the antigen, or may be expressed from the same nucleic acid vector but produced as distinct expression products.

The present invention provide a recombinant, replication-defective alphavirus-based replicon particles that encode a fusion of a polypeptide antigen of choice with an intercellular transport protein that, when expressed in a transfected cell, is capable of distributing the antigen to many surrounding cells. This has been accomplished by the use of a stable packaging cell line (PCL), which is capable of generating alphavirus replicon particles without contamination from replication-competent virus.

This invention has been exemplified using the HSV-1 VP22 protein linked to a model tumor antigen, human papillomavirus type 16 (HPV-16) E7 oncoprotein and included in a nucleic acid which is a Sindbis virus (SIN)-based replicon particle encoding the VP22-E7 fusion and using a PCL termed SIN-PCL. The linkage of VP22 to E7 in these SIN replicon particles resulted in a significant increase in the number of E7-specific $CD8^+$ T cell precursors and a strong antitumor effect against E7-expressing tumors in vaccinated C57BL/6 mice relative to wild-type E7 SIN replicon particles. Furthermore, a head-to-head comparison of VP22/E7-containing naked DNA, naked RNA replicons, or RNA replicon particle vaccines indicated that SINrep5-VP22/E7 replicon particles generated the most potent therapeutic antitumor effect. For additional disclosure, see also, Cheng, WF *et al.*, 2002, *Hum Gene Ther,* 2002, Mar;*13*:553-568, a publication by the present inventors and colleagues after the priority date of this application. Thus, the present strategy used in the context of SIN replicon particles

produce with a PCL facilitates the generation of a highly effective vaccines for widespread immunization.

The present invention is directed to a nucleic acid molecule encoding a fusion polypeptide useful as a vaccine composition, which molecule comprises:

(a) a first nucleic acid sequence encoding a first polypeptide that comprises at least one immunogenicity-potentiating polypeptide;

(b) optionally, fused in frame with the first nucleic acid sequence, a linker nucleic acid sequence encoding a linker peptide; and

(c) a second nucleic acid sequence that is linked in frame to the first nucleic acid sequence or to the linker nucleic acid sequence and that encodes an antigenic polypeptide or peptide,

which nucleic acid is in the form of a replication-defective alphavirus replicon particle prepared using a packaging cell line.

In the above nucleic acid molecule, the first polypeptide is preferably one that acts by promoting:

(a) processing of the linked antigenic polypeptide via the MHC class I pathway or targeting of a cellular compartment that increases the processing;

(b) development, accumulation or activity of antigen presenting cells or targeting of antigen to compartments of the antigen presenting cells leading to enhanced antigen presentation;

(c) intercellular transport and spreading of the antigen; or

(d) any combination of (a)-(c).

Preferably the first polypeptide is:

(a) a mycobacterial HSP70 polypeptide, the C-terminal domain thereof, or a functional homologue or derivative of the polypeptide or domain;

(b) a viral intercellular spreading protein selected from the group of herpes simplex virus-1 VP22 protein, Marek's disease virus VP22 protein or a functional homologue or derivative thereof;

(c) an endoplasmic reticulum chaperone polypeptide selected from the group of calreticulin, ER60, GRP94, gp96, or a functional homologue or derivative thereof

(d) a cytoplasmic translocation polypeptide domains of a pathogen toxin selected from the group of domain II of *Pseudomonas* exotoxin ETA (ETAdII) or a functional homologue or derivative thereof;

(e) a polypeptide that targets the centrosome compartment of a cell selected from γ-tubulin or a functional homologue or derivative thereof; or

(f) a polypeptide that stimulates dendritic cell processors or activates dendritic cell activity selected from the group of GM-CSF, Flt3-ligand extracellular domain, or a functional homologue or derivative thereof

More preferably, the first polypeptide above is selected from the group consisting of *Mycobacterium tuberculosis* HSP70, the HSP70 C-terminal domain, HSV-1 VP22, MDV VP22, calreticulin, *Pseudomonas* ETAdII, GM-CSF, Flt-3 ligand extracellular domain or γ-tubulin.

In a preferred embodiment, the first polypeptide is a transport polypeptide comprising SEQ ID NO:5 or 7 or an active fragment thereof.

In the above nucleic acid molecule, the antigenic polypeptide preferably comprises an epitope that binds to, and is presented on the cell surface by, an MHC class I protein. Preferably, the epitope is between about 8 and about 11 amino acid residues in length.

In the above nucleic acid molecule, the antigen is preferably one which is present on, or cross-reactive with an epitope of, a pathogenic organism, cell, or virus. A preferred virus is a human papilloma virus. A preferred antigen is the E7 polypeptide of HPV-16 or an antigenic fragment thereof.

In the above nucleic acid molecule, the pathogenic organism may be a bacterium.

Further, the pathogenic cell is preferably a tumor cell. In that case, the antigen is a tumor-specific or tumor-associated antigen, for example, a peptide of the HER-2/neu protein.

The above nucleic acid molecule may be operatively linked to a promoter. The promoter is preferably one which is expressed in an APC, preferably a DC.

The above nucleic acid molecule is preferably an RNA replicon wherein the alphavirus is Sindbis virus, Semliki forest virus or Venezuelan equine encephalitis virus, most preferably Sindbis virus. The nucleic acid molecule may have the sequence of the SINrep5 molecule

In the above nucleic acid molecule, the packaging cell line is preferably one in which genes encoding capsid and envelope glycoproteins of the alphavirus are separated in distinct cassettes to minimize formation of replication competent virus during replicon production. A most preferred packaging cell line is 987dlsplit #24.

Also provided herein is an expression vector comprising any of the nucleic acid molecules described above, operatively linked to (a) a promoter; and (b) optionally, additional regulatory sequences that regulate expression of the nucleic acid in a eukaryotic cell.

The present invention is also directed to a cell which has been modified to comprise the nucleic acid or expression vector as above Preferably, the cell expresses the nucleic acid molecule. Preferred cells as above are APCs, for example, a dendritic cell, a keratinocyte, a macrophage, a monocyte, a B lymphocyte, a microglial cell, an astrocyte, or an activated endothelial cell.

In another embodiment, the present invention is directed to a pharmaceutical composition capable of inducing or enhancing an antigen-specific immune response, comprising:

(a) pharmaceutically and immunologically acceptable excipient in combination with;

(b) a composition selected from the group consisting of:

(i) the above nucleic acid molecule or expression vector;

(ii) the above cell; and

(iii) any combination of (i) and (ii).

Also provided is a method of inducing or enhancing an antigen specific immune response in a subject comprising administering to the subject an effective amount of the above pharmaceutical composition, thereby inducing or enhancing the response. The response is preferably one which is mediated at least in part by $CD8^+$ cytotoxic T lymphocytes (CTL). Alternatively, or additionally, the response may be mediated at least in part by antibodies.

The present invention includes a method of inducing or enhancing an antigen specific immune response in cells or in a subject comprising administering to the cells or to the subject an effective amount of the pharmaceutical composition as above, thereby inducing or enhancing the response.

In the foregoing method, the composition may be is administered *ex vivo* to the cells. These cells may comprise APCs, such as DCs. Preferably, the APCs are human APCs. These APCs are preferably isolated from a living subject. This method may further comprising a step of administering the *ex vivo*-treated cells to a histocompatible subject. Preferably, the cells are human cells and the subject is a human.

In all the foregoing method o *in vivo* treatment, the administering is preferably by a intramuscular, intradermal, or subcutaneous route. Alternatively, when treating a tumor, the administering may be intratumoral or peritumoral.

The present invention provides a method of increasing the numbers or lytic activity of $CD8^+$ CTLs specific for a selected antigen in a subject, comprising administering to the subject an effective amount of a composition selected from the group consisting of:

(a) the nucleic acid molecule or expression vector as above;

(b) the cell as above, and

(c) any combination of (a) and (b), wherein

(i) the nucleic acid molecule, the expression vector or the cell comprises the antigen,

(ii) the antigen comprises an epitope that binds to, and is presented on the cell surface by, MHC class I proteins,

thereby increasing the numbers or activity of the CTLs.

Also provided is a method of inhibiting growth or preventing re-growth of a tumor in a subject, comprising administering to the subject, preferably intratumorally or peritumorally, an effective amount of a composition selected from the group consisting of:

(a) the above nucleic acid molecule or expression vector;

(b) the above cells; and

(c) any combination of (a) and (b), wherein

(i) the nucleic acid molecule, the expression vector or the cell comprises the antigen,

(ii) the antigen comprises one or more tumor-associated or tumor-specific epitopes present on the tumor in the subject

thereby inhibiting the growth or preventing the re-growth.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A, 1B, 1C and 1D. Immunofluorescence staining to demonstrate the expression and distribution of E7 and chimeric VP22/E7 protein. BHK 21 cells were infected with SINrep5-E7 (Fig. 1A and B) or SINrep5-VP22/E7 (Fig. 1C and D) replicon particles. Infected cells were fixed in 10% formalin and stained for HPV-16 E7 protein at 48 hrs (Fig. 1A and C) or 72 hrs (Fig. 1B and D) after infection. See Example I.. Slides were mounted and observed immediately under a fluorescence microscope. (Fig. 1A) BHK21 cells infected with SINrep5-E7

replicon particles and stained for E7 at 48 hours after infection. Note: E7 protein was predominantly located in the nucleus. Fig. 1B: BHK21 cells infected with SINrep5-E7 replicon particles and stained for E7 at 72 hours after infections. Note: E7 protein remained in the nucleus 72 hours after infection. Fig. 1C: BHK21 cells infected with SINrep5-VP22/E7 replicon particles and stained for E7 at 48 hours after infection. Note: VP22/E7 protein was mostly located in the cytoplasm. Fig. 1D: BHK21 cells infected with SINrep5-VP22/E7 replicon particles and stained for E7 at 72 hours after infections. Note: intercellular spreading of VP22/E7 protein to many neighboring cells became apparent 72 hours after infection.

Figure 2A. 2B and 2C. Intracytoplasmic cytokine staining followed by flow cytometry analysis to demonstrate that SINrep5-VP22/E7 replicon particles can enhance E7-specific $CD8^+$ but not $CD4^+$ T cell immunologic responses. Vaccination of mice and preparation of splenocytes is described in Example I. **Fig. 2A:** Representative figure of flow cytometric analysis demonstrating E7-specific $CD8^+$ T cell precursors in splenocytes from vaccinated mice. The number of IFN-γ-secreting $CD8^+$ T cell precursors is shown in the upper right corner. **Fig. 2B:** Histogram to show E7-specific IFN-γ-secreting $CD8^+$ T cell precursors in vaccinated mice. The number of IFN-γ -producing E7-specific $CD8^+$ T cells was determined using flow cytometry in the presence (solid columns) or absence (open columns) of MHC class I restricted E7 peptide (aa 49-57). Data are expressed as mean number of $CD8^+$,IFN-γ^+ cells/$3x10^5$ splenocytes; bars, SE. **Fig. 2C:** Histogram to show E7-specific IFN-γ-secreting $CD4^+$ T cell precursors. The number of IFN-γ -producing E7-specific $CD4^+$ T cells was determined using flow cytometry in the presence (solid columns) or absence (open columns) of MHC class II restricted E7 peptide (aa 30-67). Data are expressed as mean number of $CD4^+$, IFN-γ^+ cells/$3x10^5$ splenocytes; bars, SE. The intracellular cytokine staining results are from one representative experiment of two performed.

Figures 3A and 3B. Intracytoplasmic cytokine staining followed by flow cytometry analysis to demonstrate the generation of E7-specific $CD8^+$ T cell precursors using different routes of administration and dosages of the SINrep5-VP22/E7 particle vaccine. **Fig. 3A:** Mice were immunized with $5x10^6$ IU/mouse of SINrep5-VP22/E7 replicon particles via intramuscular, intraperitoneal, or subcutaneous injection. Note: The intramuscular route generated the highest number of E7-specific $CD8^+$ T cell precursors. **Fig. 3B**: Mice were immunized intramuscularly

with different dosages of SINrep5-VP22/E7 replicon particles as described in Example I. With increasing dosages of SINrep5-VP22/E7 particles, the number of E7-specific $CD8^+$ T cell precursors increased gradually, reaching a plateau at the dose of $5x10^6$ IU/mouse. The number of IFN-γ-producing E7-specific $CD8^+$ T cells was determined using flow cytometry in the presence (solid columns) or absence (open columns) of MHC class I restricted E7 peptide (aa 49-57). Data are expressed as mean number of IFN-γ-secreting $CD8^+$ T cells/$3x10^5$ splenocytes; bars, SE. The results shown here are from one representative experiment of two performed.

Figure 4. *In vivo* tumor protection experiments to demonstrate the antitumor effect generated by SINrep5 replicon particles against TC-1 tumors. Mice were immunized with various SINrep5 replicon particles as described in Example I. One week after vaccination, mice were challenged with 10^4 TC-1 cells/mouse subcutaneously and monitored for evidence of tumor growth by palpation and inspection twice a week. 100% of mice receiving the SINrep5-VP22/E7 replicon particles remained tumor-free 60 days after TC-1 challenge. All of the mice in the other vaccination groups exhibited tumor growth within 20 days after tumor challenge. The data shown here are from one representative experiment of two performed.

Figure 5A and 5B. *In vivo* tumor treatment experiments to demonstrate the antitumor effect generated by SINrep5 replicon particles against TC-1 tumors. Mice were challenged and treated as described in the Materials and Methods. **Fig. 5A:** Treatment of pulmonary nodules with SINrep5-VP22/E7 replicons relative to other SINrep5 constructs. Mice treated with SINrep5-VP22/E7 replicon particles displayed a significantly lower mean number of pulmonary nodules 3 days after tumor challenge (0.7 ± 0.3) than mice treated with the other SINrep5 replicon particle vaccines. **Fig. 5B:** Treatment of pulmonary nodules with SINrep5-VP22/E7 three, seven, and fourteen days after tumor challenge. Mice treated with SINrep5-VP22/E7 replicon particles exhibited a significantly lower mean number of pulmonary nodules three days (0.7 ± 0.3), seven days (0.5 ± 0.3), or fourteen days (25.0 ± 4.0) after tumor challenge compared to SINrep5 control (no insert) (one-way ANOVA, $P<0.05$).

Figure 6A and 6B. *In vivo* tumor treatment experiment to compare the antitumor effect in mice treated with VP22/E7 naked DNA, naked SINrep5-VP22/E7 RNA replicons, or SINrep5-VP22/E7 RNA replicon particles. Mice were challenged with TC-1 and treated with VP22/E7 naked DNA, naked SINrep5-VP22/E7 RNA replicons, or SINrep5-VP22/E7 RNA replicon particles as described in Example I. **Fig 6A:** Treatment of pulmonary tumor nodules

with various VP22/E7-containing vaccines. Mice treated with SINrep5-VP22/E7 replicon particles displayed a significantly lower mean lung weight after tumor challenge than mice treated with VP22/E7 DNA and naked SINrep5-VP22/E7 RNA replicon vaccines. **Fig 1B:** Representative gross pictures of pulmonary metastatic nodules in mice treated with the different VP22/E7-containing vaccines.

Figure 7. *In vivo* antibody depletion experiments to determine the effect of lymphocyte subsets on the potency of SINrep5-VP22/E7 replicon particles as a vaccine. Mice were immunized intramuscularly with $5x10^6$ IU/mouse of SINrep5-VP22/E7 replicon particles. CD4, CD8 and NK1.1 depletions were initiated one week after vaccination. Two weeks after vaccination, mice were challenged with 10^4 TC-1 cells/mouse subcutaneously. All naïve mice and all mice depleted of $CD8^+$ T cells grew tumors within 14 days after tumor challenge. 80% of mice depleted of $CD4^+$ T cells and 60% of mice depleted of NK1.1 cells developed tumors within 60 days after tumor challenge. Note: these results suggested that $CD8^+$ T cells, $CD4^+$ T cells and NK cells are all important for the anti-tumor immunity generated by the SINrep5-VP22/E7 replicon particles. Data from the antibody depletion experiment shown here are from one representative experiment of two performed.

FIG. 8A-8D. TUNEL assay of apoptotic cells in the skeletal muscle of vaccinated mice. These photomicrographs show mus-cle tissue at the injection sites from (A) control mice immunized with normal saline, (B) mice immunized with VP22–E7 DNA, (C) mice immunized with VP22–E7 RNA, and (D) mice immunized with SINrep5-VP22/E7 replicon particles. Vaccination with SINrep5-VP22/E7 replicon particles induced a greater degree of apoptosis in muscle tissue compared with the other groups.

Figure 9. Activity of E7-specific CTL. BHK21 cells were first infected with various SINrep5 replicon particles. Infected BHK21 cells were co-incubated with bone marrow-derived DCs. DCs were used as target cells and an E7-specific $CD8^+$ T cell line served as effector cells. CTL assays with various E:T ratios were performed. The SINrep5-VP22/E7 replicon particle vaccine generated greater cytotoxicity (measured at E:T ratios of 9 and 27 ($p<0.01$)) compared to BHK21 cells infected with SINrep5-E7 replicon particles. The CTL assays shown here are from one representative experiment of two performed.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

All references cited above and below are incorporated by reference in their entirety herein, whether specifically incorporated or not.

The invention provides compositions and methods for enhancing the immune responses, particularly cytotoxic T cell immune responses, induced by *ex vivo* or *in vivo* administration of nucleic acid vaccines that encode chimeric polypeptides. The preferred chimeric or fusion polypeptide comprises (1) at least one first polypeptide or peptide that, upon introduction to cells of the host immune system, *in vitro* or *in vivo*, promotes or potentiates immunogenicity of the second polypeptide or peptide (the antigen). For this reason, the first polypeptide has been termed an "immunogenicity-potentiating (or promoting) polypeptide, abbreviated "IPP". These are described in more detail below. The nucleic acid vaccine further comprises (2) at least one second polypeptide or peptide that is an antigenic polypeptide or peptide in the host against which it is desired to induce an immune response.

In a preferred embodiment, the chimeric or fusion polypeptides are "indirectly" administered by administration of a nucleic acid vector that encodes the chimeric molecule; the nucleic acid construct, and thus the fusion protein, is expressed *in vivo*. In the present invention, this nucleic acid construct of vector is in the form of replication-defective alphaviruses generated from stable alphavirus packaging cell lines ("PCL").

The IPP is a polypeptide that acts by promoting

(1) processing via the MHC class I pathway.

(2) development or activity of APCs or targeting of DC's for antigen presentation

(3) intercellular transport and spreading thereby conferring such properties on a linked antigenic polypeptide or peptide.

It is important to note that these categories are artificial and are being made to assist in classifying the polypeptides that are disclosed herein. A number of the polypeptides that are ascribed ton one category, act in ways that would place them in another of these categories.

IPP's that Promote Processing via the MHC Class I Pathway.

For convenience, a polypeptide or peptide that promotes processing via the MHC class I pathway is abbreviated herein as "MHC_I-PP".

One exemplary MHC_I -PP described herein is Hsp70. However, it is understood that any protein, or functional fragment or variant thereof, that has this activity can be used in the invention. A preferred fragment is a C-terminal domain ("CD") of Hsp70, which is designated "$Hsp70_{CD}$". One $Hsp70_{CD}$ spans from about residue 312 to the C terminus of Hsp70 (SEQ ID NO:9). A preferred shorter polypeptide spans from about residue 517 to the C-terminus of SEQ ID NO:9. Shorter peptides from that sequence that have the ability to promote protein processing via the MHC-1 class I pathway are also included, and may be defined by routine experimentation.

Another category of MHC_I-PP is an ER chaperone polypeptide such as calreticulin, ER60, GRP94 or gp96, well-characterized ER chaperone polypeptides that representatives of the HSP90 family of stress-induced proteins (Argon (1999) *Semin. Cell Dev..Biol.* 10:495-505; Sastry (1999) *J.* Biol. Chem. *274*:12023-12035; Nicchitta (1998) *Curr. Opin. Immunol.* 10:103-109; U.S. Patent 5,981,706))

Another group of proteins that act as MHC_I-PP are cytoplasmic translocation polypeptide domains of pathogen toxins, such as domain II of *Pseudomonas* exotoxin ETA (ETAdII) or of similar toxins from *Diptheria, Clostridium, Botulinum, Bacillus, Yersinia, Vibrio cholerae,* or *Bordetella pertussis*; or active fragments or domains of any of the foregoing polypeptides.

Polypeptides that route a linked protein to the cell centrosome compartment promote processing for antigen presentation. Thus, linkage of γ-tubulin to an antigen (E7 protein) efficiently re-routed E7 into the centrosome compartment, making γ-tubulin a useful IPP according to this invention.

IPP's that Promote Development or Activity of APCs or Targeting of DC

For convenience, a polypeptide or peptide that promotes development or activity of APCs or targeting of APCs, , preferably DC's, is termed a "DC-PP".

One class of such IPPs are immunostimulatory cytokines that target APCs, primarily eferably DC's, such as granulocyte macrophage colony stimulating factor (GM-CSF), or active fragments or domains thereof. DNA encoding the cytokine GM-CSF gene to DNA encoding an antigen (*e.g.*, an HIV or hepatitis C antigen) enhanced the potency of DNA vaccines (Lee, AH *et al., Vaccine 17*: 473-9, 1999; Lee, SW *et al., J Virol. 72*: 8430-6, 1998). The chimeric GM-CSF/antigen is believed to act as an immunostimulatory signal to DCs, inducing their

differentiation from an immature form (Banchereau, J *et al., Nature* 392: 245-52, 1998). Since DCs and their precursors express high levels of GM-CSF receptors, the chimeric GM-CSF/antigen should target and concentrate the linked antigen to the DCs and further improve the vaccine's potency.

The Flt-3 ligand (FL) stimulates growth of DC precursors. Thus, the constructs of the present invention include FL, preferably its ECD. FL also targets a linked antigen to DCs thereby promoting antigen presentation.

The APCs targeted by the compositions of the present invention jnclude DCs keratinocytes, astrocytes, monocytes, macrophages, B lymphocytes, a microglial cell, or activated endothelial cells, and the like, although DC are a preferred target..

IPPs that Promote Intercellular Transport and Spread

Examples of such proteins are VP22, a herpes simplex virus type 1 (HSV-1) protein and its "homologues" in other herpes viruses, such as the avian Marek's Disease Virus (MDV) have the property of intercellular transport that provide an approach for enhancing vaccine potency. In commonly assigned patent application WO 02/09645, published 07-FEB-02 (Wu, TC *et al.*, filed 01-AUG-01) incorporated by reference herein, and in several publications with their colleagues (Hung, CF *et al.*, 2002, *J Virol 76*:2676-2682; Hung, CF *et al.*, 2001, *J Immunol;166*:5733-5744; Cheng, WF *et al.*, 2001, *J Virol. 75*:2368-2376), the present inventors disclosed novel fusions of VP22 and its homologues with a model antigen, human papillomavirus type 16 (HPV-16) E7, in a DNA vaccine which generated enhanced spreading and MHC class I presentation of antigen. These properties led to a dramatic increase in the number of E7-specific $CD8^+$ T cell precursors in vaccinated mice (at least 50-fold) and converted a less effective DNA vaccine into one with significant potency against E7-expressing tumors. In comparison, a non-spreading mutant,VP22(1-267), failed to enhance vaccine potency. Thus the potency of DNA vaccines was dramatically improved through enhanced intercellular spreading and MHC class I presentation of the antigen.

Despite the limited identity between the amino acid sequence of MDV-1 UL49 (VP22) and that of HSV-1 VP22 -- approximately 20% -- both polypeptides enhanced DNA vaccine potency when linked to a "model" antigen, E7 9, as disclosed by the present inventors in WO 02/09645. It is important to note that not all molecules with "trafficking properties" have this

action of enhancing vaccine potency. The present inventors found, when analyzing naked DNA vaccines comprising E7 DNA fused to DNA encoding sequences derived from proteins with trafficking properties such as HIV TAT protein, the membrane-translocating sequence and the third helix of the Antennapedia homeodomain did not generate CD8+ T cell- responses of similar potency as those induced by VP22/E7. Therefore, they concluded that VP22 and homologues thereof have a unique property or properties that distinguish them from these other constructs.

The order in which the two (or more) component polypeptides of the present fusion protein of this invention are arranged, and therefore, the order of the encoding nucleic acid fragments in the nucleic acid vector, can be altered without affecting immunogenicity of the fusion polypeptides proteins and the utility of the composition. For example, the Hsp70-encoding (or FL -encoding) DNA sequences may be located 5' or 3' to the target antigen-encoding sequences. In one embodiment, these polypeptide-encoding nucleic acid domains are in-frame so that the DNA construct encodes a recombinant fusion polypeptide in which the antigen is located N- terminal to the Hsp70 or FL derived polypeptide.

The vaccines of the present invention include, the antigenic epitope itself and an IPP, such as an MHC_I-PP like Hsp70 or its active domain (CD), or a DC-PP such as FL, or a intercellular spreading protein such as VP22, as summarized above and describe in more detail below.

In fact, the vaccine construct of the present invention optionally, may also include more than one of the foregoing IPPs. Another useful polypeptide for the present constructs is a costimulatory signal, such as a B7 family protein, including B7-DC (see commonly assigned U.S. patent application Serial No. 09/794,210), B7.1, B7.2, soluble CD40, *etc.*).

For description of some of the foregoing, see, for example, commonly owned International patent publications WO 01/29233 (26-APR-01), WO 02/09645 (07-FEB-02) and WO 02/12281 (14-FEB-02).

The Examples described herein demonstrate that the methods of the invention can enhance a cellular immune response, particularly, tumor-destructive CTL reactivity, induced by a DNA vaccine encoding an epitope of a human pathogen. Human HPV-16 E7 was used as a model antigen for vaccine development because human papillomaviruses (HPVs), particularly

HPV-16, are associated with most human cervical cancers. The oncogenic HPV protein E7 is important in the induction and maintenance of cellular transformation and co-expressed in most HPV-containing cervical cancers and their precursor lesions. Therefore, cancer vaccines, such as the compositions of the invention, that target E7 can be used to control of HPV-associated neoplasms (Wu (1994) *Curr. Opin. Immunol. 6*:746-754).

Unless defined otherwise, all known technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art of this invention. As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

The term "antigen" or "immunogen" as used herein refers to a compound or composition comprising a peptide, polypeptide or protein which is "antigenic" or "immunogenic" when administered (or expressed *in vivo* by an administered nucleic acid, *e.g.*, a DNA vaccine) in an appropriate amount (an "immunogenically effective amount"), *i.e.*, capable of inducing, eliciting, augmenting or boosting a cellular and/or humoral immune response either alone or in combination or linked or fused to another substance (which can be administered at once or over several intervals). An immunogenic composition can comprise an antigenic peptide of at least about 5 amino acids, a peptide of 10 amino acids in length, a polypepide fragment of 15 amino acids in length, 20 amino acids in length or longer. Smaller immunogens may require presence of a "carrier" polypeptide *e.g.*, as a fusion protein, aggregate, conjugate or mixture, preferablyl linked (chemically or otherwise) to the immunogen. The immunogen can be recombinantly expressed from a vaccine vector, which can be naked DNA comprising the immunogen's coding sequence operably linked to a promoter, *e.g.*, an expression cassette as described herein. The immunogen includes one or more antigenic determinants or epitopes which may vary in size from about 3 to about 15 amino acids.

The term "epitope" as used herein refers to an antigenic determinant or antigenic site that interacts with an antibody or a T cell receptor (TCR), *e.g.*, the MHC class I-binding peptide compositions (or expressed products of the nucleic acid compositionsof the invention) used in the methods of the invention. An "antigen" is a molecule or chemical structure that either induces an immune response or is specifically recognized or bound by the product or mediator of an immune response, such as an antibody or a CTL. The specific conformational or stereochemical "domain" to which an antibody or a TCR bind is an "antigenic determinant" or

"epitope." TCRs bind to peptide epitopes which are physically associated with a third molecule, a major histocompatibility complex (MHC) class I or class II protein.

The term "recombinant" refers to (1) a nucleic acid or polynucleotide synthesized or otherwise manipulated *in vitro*, (2) methods of using recombinant DNA technology to produce gene products in cells or other biological systems, or (3) a polypeptide encoded by a recombinant nucleic acid. For example, the FL-encoding nucleic acid or polypeptide, the nucleic acid encoding an MHC class I-binding peptide epitope (antigen) or the peptide itself can be rececombinant. "Recombinant means" includes ligation of nucleic acids having various coding regions or domains or promoter sequences from different sources into a single unit in the form of an expression cassette or vector for expression of the coding sequences in the vectors resulting in production of the encoded polypeptide.

Specifically, the present inventors investigated the novel use of VP22 proteins linked to a model antigen (HPV-16 E7) in the context of a PCL-generated replication-defective Sinbis virus replicon vaccine and found that it led to the spread of linked antigen to surrounding cells and enhanced antigen-specific immune responses and antitumor effects.

The following sections provide various nucleic acid and amino acid sequences of a model antigenic (HPV16-E7) protein and various of the IPP's as listed above.

The "wild-type" amino acid sequence of HPV-E7 protein is provided below:

MHGDTPTLHEYMLDLQPETTDLYCYEQLNDSSEEEDEIDGPAGQAEPDRAHYNIVTFCCKCDST
LRLCVQSTHVDIRTLEDLLMGTLGIVCPICSQKP

(SEQ ID NO:1), GENBANK Accession No. AAD33353.

Production of various vectors may result in loss of certain residues of the antigen without affecting the immunogenicity of the vaccine and the specificity of the immune response. For example, the present inventors have described elsewhere a pcDNA3 naked DNA vector wherein only 96 of the 98 residues of E7 are present and the C-terminal two residues of wild-type E7, Lys and Pro are absent from this contstruct. This is an example of a deletion variant. Such deletion variants (*e.g.*, terminal truncation of two or a small number of amino acids) of other antigenic polypeptides are examples of the embodiments intended within the scope of the fusion polypeptides of this invention.

Such a modified HPV-E7 (nucleic acid sequence is SEQ ID NO:2; amino acid sequence is SEQ ID NO:3) is shown below:

```
1/1                                                 31/11
atg cat gga gat aca cct aca ttg cat gaa tat atg tta gat ttg caa cca gag aca act
Met his gly asp thr pro thr leu his glu tyr met leu asp leu gln pro glu thr thr
61/21                                               91/31
gat ctc tac tgt tat gag caa tta aat gac agc tca gag gag gag gat gaa ata gat ggt
asp leu tyr cys tyr glu gln leu asn asp ser ser glu glu glu asp glu ile asp gly
121/41                                              151/51
cca gct gga caa gca gaa ccg gac aga gcc cat tac aat att gta acc ttt tgt tgc aag
pro ala gly gln ala glu pro asp arg ala his tyr asn ile val thr phe cys cys lys
181/61                                              211/71
tgt gac tct acg ctt cgg ttg tgc gta caa agc aca cac gta gac att cgt act ttg gaa
cys asp ser thr leu arg leu cys val gln ser thr his val asp ile arg thr leu glu
241/81                                              271/91
gac ctg tta atg ggc aca cta gga att gtg tgc ccc atc tgt tct cag gat aag ctt
asp leu leu met gly thr leu gly ile val cys pro ile cys ser gln asp lys leu
```

After cloning, the last 9 nucleotides (3 amino acids were modified, as shown above underscored and/or bold. The original GENBANK sequence shown above has a Lys-Pro after the Gln in position 96 (encoded by `aaa/cca/taa`, rather than Asp-Lys-Leu (encoded by `gat/aag/ctt`).

A preferred intercellular spreading protein is preferably a viral spreading protein, most preferably a herpesvirus VP22 protein. Exemplified herein are fusion constructs that comprise herpes simplex virus-1 (HSV-1) VP22 (abbreviated HVP22). Also shown below is its homologue from Marek's disease virus (MDV) termed MDV-VP22 or MVP-22). Also included in the invention are homologues of VP22 from other members of the herpesviridae or polypeptides from nonviral sources that are considered to be homologous and share the functional characteristic of promoting intercellular spreading of a polypeptide or peptide that is fused or chemically conjugated thereto.

DNA encoding HVP22 has the sequence SEQ ID NO:4 which is shown as nucleotides 1-903 below. The VP22 amino acid sequence (1-301) is SEQ ID NO:5.

```
1/1                                                 31/11
ATG ACC TCT CGC CGC TCC GTG AAG TCG GGT CCG CGG GAG GTT CCG CGC GAT GAG TAC GAG
Met thr ser arg arg ser val lys ser gly pro arg glu val pro arg asp glu tyr glu
61/21                                               91/31
GAT CTG TAC TAC ACC CCG TCT TCA GGT ATG GCG AGT CCC GAT AGT CCG CCT GAC ACC TCC
asp leu tyr tyr thr pro ser ser gly met ala ser pro asp ser pro pro asp thr ser
121/41                                              151/51
CGC CGT GGC GCC CTA CAG ACA CGC TCG CGC CAG AGG GGC GAG GTC CGT TTC GTC CAG TAC
arg arg gly ala leu gln thr arg ser arg gln arg gly glu val arg phe val gln tyr
181/61                                              211/71
GAC GAG TCG GAT TAT GCC CTC TAC GGG GGC TCG TCT TCC GAA GAC GAC GAA CAC CCG GAG
asp glu ser asp tyr ala leu tyr gly gly ser ser ser glu asp asp glu his pro glu
241/81                                              271/91
GTC CCC CGG ACG CGG CGT CCC GTT TCC GGG GCG GTT TTG TCC GGC CCG GGG CCT GCG CGG
val pro arg thr arg arg pro val ser gly ala val leu ser gly pro gly pro ala arg
301/101                                             331/111
GCG CCT CCG CCA CCC GCT GGG TCC GGA GGG GCC GGA CGC ACA CCC ACC ACC GCC CCC CGG
ala pro pro pro pro ala gly ser gly gly ala gly arg thr pro thr thr ala pro arg
361/121                                             391/131
GCC CCC CGA ACC CAG CGG GTG GCG TCT AAG GCC CCC GCG GCC CCG GCG GCG GAG ACC ACC
ala pro arg thr gln arg val ala ser lys ala pro ala ala pro ala ala glu thr thr
421/141                                             451/151
CGC GGC AGG AAA TCG GCC CAG CCA GAA TCC GCC GCA CTC CCA GAC GCC CCC GCG TCG ACG
arg gly arg lys ser ala gln pro glu ser ala ala leu pro asp ala pro ala ser thr
```

```
481/161                                          511/171
GCG CCA ACC CGA TCC AAG ACA CCC GCG CAG GGG CTG GCC AGA AAG CTG CAC TTT AGC ACC
ala pro thr arg ser lys thr pro ala gln gly leu ala arg lys leu his phe ser thr
541/181                                          571/191
GCC CCC CCA AAC CCC GAC GCG CCA TGG ACC CCC CGG GTG GCC GGC TTT AAC AAG CGC GTC
ala pro pro asn pro asp ala pro trp thr pro arg val ala gly phe asn lys arg val
601/201                                          631/211
TTC TGC GCC GCG GTC GGG CGC CTG GCG GCC ATG CAT GCC CGG ATG GCG GCT GTC CAG CTC
phe cys ala ala val gly arg leu ala ala met his ala arg met ala ala val gln leu
661/221                                          691/231
TGG GAC ATG TCG CGT CCG CGC ACA GAC GAA GAC CTC AAC GAA CTC CTT GGC ATC ACC ACC
trp asp met ser arg pro arg thr asp glu asp leu asn glu leu leu gly ile thr thr
721/241                                          751/251
ATC CGC GTG ACG GTC TGC GAG GGC AAA AAC CTG CTT CAG CGC GCC AAC GAG TTG GTG AAT
ile arg val thr val cys glu gly lys asn leu leu gln arg ala asn glu leu val asn
781/261                                          811/271
CCA GAC GTG GTG CAG GAC GTC GAC GCG GCC ACG GCG ACT CGA GGG CGT TCT GCG GCG TCG
pro asp val val gln asp val asp ala ala thr ala thr arg gly arg ser ala ala ser
841/281                                          871/291
CGC CCC ACC GAG CGA CCT CGA GCC CCA GCC CGC TCC GCT TCT CGC CCC AGA CGG CCC GTC
arg pro thr glu arg pro arg ala pro ala arg ser ala ser arg pro arg arg pro val
901/301
GAG
glu
```

DNA encoding MVP22 is SEQ ID NO:6 shown below:

```
atg ggg gat tct gaa agg cgg aaa tcg gaa cgg cgt cgt tcc ctt gga   48
tat ccc tct gca tat gat gac gtc tcg att cct gct cgc aga cca tca   96
aca cgt act cag cga aat tta aac cag gat gat ttg tca aaa cat gga   144
cca ttt acc gac cat cca aca caa aaa cat aaa tcg gcg aaa gcc gta   192
tcg gaa gac gtt tcg tct acc acc cgg ggt ggc ttt aca aac aaa ccc   240
cgt acc aag ccc ggg gtc aga gct gta caa agt aat aaa ttc gct ttc   288
agt acg gct cct tca tca gca tct agc act tgg aga tca aat aca gtg   336
gca ttt aat cag cgt atg ttt tgc gga gcg gtt gca act gtg gct caa   384
tat cac gca tac caa ggc gcg ctc gcc ctt tgg cgt caa gat cct ccg   432
cga aca aat gaa gaa tta gat gca ttt ctt tcc aga gct gtc att aaa   480
att acc att caa gag ggt cca aat ttg atg ggg gaa gcc gaa acc tgt   528
gcc cgc aaa cta ttg gaa gag tct gga tta tcc cag ggg aac gag aac   576
gta aag tcc aaa tct gaa cgt aca acc aaa tct gaa cgt aca aga cgc   624
ggc ggt gaa att gaa atc aaa tcg cca gat ccg gga tct cat cgt aca   672
cat aac cct cgc act ccc gca act tcg cgt cgc cat cat tca tcc gcc   720
cgc gga tat cgt agc agt gat agc gaa taa                          747
```

The amino acid sequence of the MDV PV22, SEQ ID NO:7, is shown below:

```
Met Gly Asp Ser Glu Arg Arg Lys Ser Glu Arg Arg Arg Ser Leu Gly   16
Tyr Pro Ser Ala Tyr Asp Asp Val Ser Ile Pro Ala Arg Arg Pro Ser   32
Thr Arg Thr Gln Arg Asn Leu Asn Gln Asp Asp Leu Ser Lys His Gly   48
Pro Phe Thr Asp His Pro Thr Gln Lys His Lys Ser Ala Lys Ala Val   64
Ser Glu Asp Val Ser Ser Thr Thr Arg Gly Gly Phe Thr Asn Lys Pro   80
Arg Thr Lys Pro Gly Val Arg Ala Val Gln Ser Asn Lys Phe Ala Phe   96
Ser Thr Ala Pro Ser Ser Ala Ser Ser Thr Trp Arg Ser Asn Thr Val  112
Ala Phe Asn Gln Arg Met Phe Cys Gly Ala Val Ala Thr Val Ala Gln  128
Tyr His Ala Tyr Gln Gly Ala Leu Ala Leu Trp Arg Gln Asp Pro Pro  144
Arg Thr Asn Glu Glu Leu Asp Ala Phe Leu Ser Arg Ala Val Ile Lys  160
Ile Thr Ile Gln Glu Gly Pro Asn Leu Met Gly Glu Ala Glu Thr Cys  176
Ala Arg Lys Leu Leu Glu Glu Ser Gly Leu Ser Gln Gly Asn Glu Asn  192
Val Lys Ser Lys Ser Glu Arg Thr Thr Lys Ser Glu Arg Thr Arg Arg  208
Gly Gly Glu Ile Glu Ile Lys Ser Pro Asp Pro Gly Ser His Arg Thr  224
His Asn Pro Arg Thr Pro Ala Thr Ser Arg Arg His His Ser Ser Ala  240
Arg Gly Tyr Arg Ser Ser Asp Ser Glu  -                           249
```

The sequences of Hsp70 from *M. tuberculosis* is shown below

(nucleic acid is SEQ ID NO:8; amino acids are SEQ ID NO:9)

```
1/1                                                    31/11
atg gct cgt gcg gtc ggg atc gac ctc ggg acc acc aac tcc gtc gtc tcg gtt ctg gaa
Met ala arg ala val gly ile asp leu gly thr thr asn ser val val ser val leu glu
61/21                                                  91/31
ggt ggc gac ccg gtc gtc gtc gcc aac tcc gag ggc tcc agg acc acc ccg tca att gtc
gly gly asp pro val val val ala asn ser glu gly ser arg thr thr pro ser ile val
121/41                                                 151/51
gcg ttc gcc cgc aac ggt gag gtg ctg gtc ggc cag ccc gcc aag aac cag gca gtg acc
ala phe ala arg asn gly glu val leu val gly gln pro ala lys asn gln ala val thr
181/61                                                 211/71
aac gtc gat cgc acc gtg cgc tcg gtc aag cga cac atg ggc agc gac tgg tcc ata gag
asn val asp arg thr val arg ser val lys arg his met gly ser asp trp ser ile glu
241/81                                                 271/91
att gac ggc aag aaa tac acc gcg ccg gag atc agc gcc cgc att ctg atg aag ctg aag
ile asp gly lys lys tyr thr ala pro glu ile ser ala arg ile leu met lys leu lys
301/101                                                331/111
cgc gac gcc gag gcc tac ctc ggt gag gac att acc gac gcg gtt atc acg acg ccc gcc
arg asp ala glu ala tyr leu gly glu asp ile thr asp ala val ile thr thr pro ala
361/121                                                391/131
tac ttc aat gac gcc cag cgt cag gcc acc aag gac gcc ggc cag atc gcc ggc ctc aac
tyr phe asn asp ala gln arg gln ala thr lys asp ala gly gln ile ala gly leu asn
421/141                                                451/151
gtg ctg cgg atc gtc aac gag ccg acc gcg gcc gcg ctg gcc tac ggc ctc gac aag ggc
val leu arg ile val asn glu pro thr ala ala ala leu ala tyr gly leu asp lys gly
481/161                                                511/171
gag aag gag cag cga atc ctg gtc ttc gac ttg ggt ggt ggc act ttc gac gtt tcc ctg
glu lys glu gln arg ile leu val phe asp leu gly gly gly thr phe asp val ser leu
541/181                                                571/191
ctg gag atc ggc gag ggt gtg gtt gag gtc cgt gcc act tcg ggt gac aac cac ctc ggc
leu glu ile gly glu gly val val glu val arg ala thr ser gly asp asn his leu gly
601/201                                                631/211
ggc gac gac tgg gac cag cgg gtc gtc gat tgg ctg gtg gac aag ttc aag ggc acc agc
gly asp asp trp asp gln arg val val asp trp leu val asp lys phe lys gly thr ser
661/221                                                691/231
ggc atc gat ctg acc aag gac aag atg gcg atg cag cgg ctg cgg gaa gcc gcc gag aag
gly ile asp leu thr lys asp lys met ala met gln arg leu arg glu ala ala glu lys
721/241                                                751/251
gca aag atc gag ctg agt tcg agt cag tcc acc tcg atc aac ctg ccc tac atc acc gtc
ala lys ile glu leu ser ser ser gln ser thr ser ile asn leu pro tyr ile thr val
781/261                                                811/271
gac gcc gac aag aac ccg ttg ttc tta gac gag cag ctg acc cgc gcg gag ttc caa cgg
asp ala asp lys asn pro leu phe leu asp glu gln leu thr arg ala glu phe gln arg
841/281                                                871/291
atc act cag gac ctg ctg gac cgc act cgc aag ccg ttc cag tcg gtg atc gct gac acc
ile thr gln asp leu leu asp arg thr arg lys pro phe gln ser val ile ala asp thr
901/301                                                931/311
ggc att tcg gtg tcg gag atc gat cac gtt gtg ctc gtg ggt ggt tcg acc cgg atg ccc
gly ile ser val ser glu ile asp his val val leu val gly gly ser thr arg met pro
961/321                                                991/331
gcg gtg acc gat ctg gtc aag gaa ctc acc ggc ggc aag gaa ccc aac aag ggc gtc aac
ala val thr asp leu val lys glu leu thr gly gly lys glu pro asn lys gly val asn
1021/341                                               1051/351
ccc gat gag gtt gtc gcg gtg gga gcc gct ctg cag gcc ggc gtc ctc aag ggc gag gtg
pro asp glu val val ala val gly ala ala leu gln ala gly val leu lys gly glu val
1081/361                                               1111/371
aaa gac gtt ctg ctg ctt gat gtt acc ccg ctg agc ctg ggt atc gag acc aag ggc ggg
lys asp val leu leu leu asp val thr pro leu ser leu gly ile glu thr lys gly gly
1141/381                                               1171/391
gtg atg acc agg ctc atc gag cgc aac acc acg atc ccc acc aag cgg tcg gag act ttc
val met thr arg leu ile glu arg asn thr thr ile pro thr lys arg ser glu thr phe
1201/401                                               1231/411
acc acc gcc gac gac aac caa ccg tcg gtg cag atc cag gtc tat cag ggg gag cgt gag
thr thr ala asp asp asn gln pro ser val gln ile gln val tyr gln gly glu arg glu
1261/421                                               1291/431
atc gcc gcg cac aac aag ttg ctc ggg tcc ttc gag ctg acc ggc atc ccg ccg gcg ccg
ile ala ala his asn lys leu leu gly ser phe glu leu thr gly ile pro pro ala pro
1321/441                                               1351/451
cgg ggg att ccg cag atc gag gtc act ttc gac atc gac gcc aac ggc att gtg cac gtc
arg gly ile pro gln ile glu val thr phe asp ile asp ala asn gly ile val his val
```

```
1381/461                                    1411/471
acc gcc aag gac aag ggc acc ggc aag gag aac acg atc cga atc cag gaa ggc tcg ggc
thr ala lys asp lys gly thr gly lys glu asn thr ile arg ile gln glu gly ser gly
1441/481                                    1471/491
ctg tcc aag gaa gac att gac cgc atg atc aag gac gcc gaa gcg cac gcc gag gag gat
leu ser lys glu asp ile asp arg met ile lys asp ala glu ala his ala glu glu asp
1501/501                                    1531/511
cgc aag cgt cgc gag gag gcc gat gtt cgt aat caa gcc gag aca ttg gtc tac cag acg
arg lys arg arg glu glu ala asp val arg asn gln ala glu thr leu val tyr gln thr
1561/521                                    1591/531
gag aag ttc gtc aaa gaa cag cgt gag gcc gag ggt ggt tcg aag gta cct gaa gac acg
glu lys phe val lys glu gln arg glu ala glu gly gly ser lys val pro glu asp thr   540
1621/541                                    1651/551
ctg aac aag gtt gat gcc gcg gtg gcg gaa gcg aag gcg gca ctt ggc gga tcg gat att
leu asn lys val asp ala ala val ala glu ala lys ala ala leu gly gly ser asp ile    560
1681/561                                    1711/571
tcg gcc atc aag tcg gcg atg gag aag ctg ggc cag gag tcg cag gct ctg ggg caa gcg
ser ala ile lys ser ala met glu lys leu gly gln glu ser gln ala leu gly gln ala    580
1741/581                                    1771/591
atc tac gaa gca gct cag gct gcg tca cag gcc act ggc gct gcc cac ccc ggc tcg gct
ile tyr glu ala ala gln ala ala ser gln ala thr gly ala ala his pro gly ser ala
1801/601
gat gaA AGC
asp glu ser
```

GENBANK Z95324 AL123456. This protein is encoded by nucleotides 10633-12510 of *Mycobacterium tuberculosis* genome). As a result of cloning, this was modified from the original GENBANK sequence which had at its 3' end:

ggc gag ccg ggc ggt gcc cac ccc ggc tcg gct gat gac gtt gtg gac gcg
gag gtg gtc gac gac ggc cgg gag gcc aag (SEQ ID NO:10)

which was replaced in the cloned version described above by tcg gct gat gaa agc (SEQ ID NO:11) which is bold and underlined above.

The unmodified GENBANK nucleotide sequence encoding HSP70, SEQ ID NO:12 (Accession numbers Z95324 and AL123456, is

```
  atggctcg tgcggtcggg atcgacctcg ggaccaccaa ctccgtcgtc tcggttctgg
aaggtggcga cccggtcgtc gtcgccaact ccgagggctc caggaccacc ccgtcaattg
tcgcgttcgc ccgcaacggt gaggtgctgg tcggccagcc cgccaagaac caggcagtga
ccaacgtcga tcgcaccgtg cgctcggtca agcgacacat gggcagcgac tggtccatag
agattgacgg caagaaatac accgcgccgg agatcagcgc ccgcattctg atgaagctga
agcgcgacgc cgaggcctac ctcggtgagg acattaccga cgcggttatc acgacgcccg
cctacttcaa tgacgcccag cgtcaggcca ccaaggacgc cggccagatc gccggcctca
acgtgctgcg gatcgtcaac gagccgaccg cggccgcgct ggcctacggc ctcgacaagg
gcgagaagga gcagcgaatc ctggtcttcg acttgggtgg tggcactttc gacgtttccc
tgctggagat cggcgagggt gtggttgagg tccgtgccac ttcgggtgac aaccacctcg
gcggcgacga ctgggaccag cgggtcgtcg attggctggt ggacaagttc aagggcacca
gcggcatcga tctgaccaag gacaagatgg cgatgcagcg gctgcgggaa gccgccgaga
aggcaaagat cgagctgagt tcgagtcagt ccacctcgat caacctgccc tacatcaccg
tcgacgccga caagaacccg ttgttcttag acgagcagct gacccgcgcg gagttccaac
ggatcactca ggacctgctg gaccgcactc gcaagccgtt ccagtcggtg atcgctgaca
ccggcatttc ggtgtcggag atcgatcacg ttgtgctcgt gggtggttcg acccggatgc
ccgcggtgac cgatctggtc aaggaactca ccggcggcaa ggaacccaac aagggcgtca
accccgatga ggttgtcgcg gtgggagccg ctctgcaggc cggcgtcctc aagggcgagg
tgaaagacgt tctgctgctt gatgttaccc cgctgagcct gggtatcgag accaagggcg
gggtgatgac caggctcatc* gagcgcaaca ccacgatccc caccaagcgg tcggagactt
tcaccaccgc cgacgacaac caaccgtcgg tgcagatcca ggtctatcag ggggagcgtg
```

agatcgccgc gcacaacaag ttgctcgggt ccttcgagct gaccggcatc ccgccggcgc
cgcgggggat tccgcagatc gaggtcactt tcgacatcga cgccaacggc attgtgcacg
tcaccgccaa ggacaagggc accggcaagg agaacacgat ccgaatccag gaaggctcgg
gcctgtccaa ggaagacatt gaccgcatga tcaaggacgc cgaagcgcac gccgaggagg
atcgcaagcg tcgcgaggag gccgatgttc gtaatcaagc cgagacattg gtctaccaga
cggagaagtt cgtcaaagaa cagcgtgagg ccgagggtgg ttcgaaggta cctgaagaca
cgctgaacaa ggttgatgcc gcggtggcgg aagcgaaggc ggcacttggc ggatcggata
tttcggccat caagtcggcg atggagaagc tgggccagga gtcgcaggct ctggggcaag
cgatctacga agcagctcag gctgcgtcac aggccactgg cgctgcccac cccggcggcg
agccgggcgg tgcccacccc ggctcggctg atgacgttgt ggacgcggag gtggtcgacg
acggccggga ggccaagtga

The cDNA sequence of Mouse GM-CSF (SEQ ID NO:13) is as follows:

gagctcagca agcgctctcc cccaattccc ttagccaaag tggacgccac cgaacagaca 61
gacctaggct aagaggtttg atgtctctgg ctacccgact ttgaaaattt tccgcaaagg 121
aaggcctttt gactaca***atg*** gcccacgaga gaaaggctaa ggtcctgagg aggatgtggc 181
tgcagaattt acttttcctg ggcattgtgg tctacagcct ctcagcaccc acccgctcac 241
ccatcactgt cacccggcct tggaagcatg tagaggccat caaagaagcc ctgaacctcc 301
tggatgacat gcctgtcaca ttgaatgaag aggtagaagt cgtctctaac gagttctcct 361
tcaagaagct aacatgtgtg cagacccgcc tgaagatatt cgagcagggt ctacggggca 421
atttcaccaa actcaagggc gccttgaaca tgacagccag ctactaccag acatactgcc 481
ccccaactcc ggaaacggac tgtgaaacac aagttaccac ctatgcggat ttcatagaca 541
gccttaaaac ctttctgact gatatcccct ttgaatgcaa aaaaccagtc caaaaa***tga***g 601
gaagcccagg ccagctctga atccagcttc tcagactgct gcttttgtgc ctgcgtaatg 661
agccaggaac tcggaatttc tgccttaaag ggaccaagag atgtggcaca gccacagttg 721
gagggcagta tagccctctg aaaacgctga ctcagcttgg acagcggcaa gacaaacgag 781
agatattttc tactgatagg gaccattata tttatttata tatttatatt ttttaaatat 841
ttatttattt atttatttaa ttttgcaact ctatttattg agaatgtctt accagataat 901
aaattattaa aacttt

GENBANK Accession No. X02333; start sites and termination codon are italicized and bolded.

For additional mRNA for murine GM-CSF, See GENBANK # X05906

The amino acid sequence of mouse GM-CSF (161 residues) (SEQ ID NO:14) is :

MWLQNLLFLG IVVYSLSAPT RSPITVTRPW KHVEAIKEAL NLLDDMPVTL NEEVEVVSNE
FSFKKLTCVQ TRLKIFEQGL RGNFTKLKGA LNMTASYYQT YCPPTPETDC ETQVTTYADF
IDSLKTFLTD IPFECKKPVQ K GENBANK Accession No. X02333.

A cDNA sequence encoding Pseudomonas exotoxin A (ETA) (SEQ ID NO:15) is

ctgcagctgg tcaggccgtt tccgcaacgc ttgaagtcct ggccgatata ccggcagggc 61
cagccatcgt tcgacgaata aagccacctc agccatgatg ccctttccat ccccagcgga 121
accccgacat ggacgccaaa gccctgctcc tcggcagcct ctgcctggcc gccccattcg 181
ccgacgcggc gacgctcgac aatgctctct ccgcctgcct cgccgcccgg ctcggtgcac 241
cgcacacggc ggagggccag ttgcacctgc cactcaccct tgaggcccgg cgctccaccg 301
gcgaatgcgg ctgtacctcg gcgctggtgc gatatcggct gctggccagg ggcgccagcg 361
ccgacagcct cgtgcttcaa gagggctgct cgatagtcgc caggacacgc cgcgcacgct 421
gaccctggcg gcggacgccg gcttggcgag cggccgcgaa ctggtcgtca ccctgggttg 481
tcaggcgcct gactgacagg ccgggctgcc accaccaggc cgagatggac gccctgcatg 541
tatcctccga tcggcaagcc tcccgttcgc acattcacca ctctgcaatc cagttcataa 601
atcccataaa agccctcttc cgctccccgc cagcctcccc gcatcccgca ccctagacgc 661
cccgccgctc tccgccggct cgcccgacaa gaaaaaccaa ccgctcgatc agcctcatcc 721
ttcacccatc acaggagcca tcgcgatgca cctgataccc cattggatcc ccctggtcgc 781
cagcctcggc ctgctcgccg gcggctcgtc cgcgtccgcc gccgaggaag ccttcgacct 841

```
ctggaacgaa tgcgccaaag cctgcgtgct cgacctcaag gacggcgtgc gttccagccg 901
catgagcgtc gacccggcca tcgccgacac caacggccag ggcgtgctgc actactccat 961
ggtcctggag ggcggcaacg acgcgctcaa gctggccatc gacaacgccc tcagcatcac 1021
cagcgacggc ctgaccatcc gcctcgaagg cggcgtcgag ccgaacaagc cggtgcgcta 1081
cagctacacg cgccaggcgc gcggcagttg gtcgctgaac tggctggtac cgatcggcca 1141
cgagaagccc tcgaacatca aggtgttcat ccacgaactg aacgccggca accagctcag 1201
ccacatgtcg ccgatctaca ccatcgagat gggcgacgag ttgctggcga agctggcgcg 1261
cgatgccacc ttcttcgtca gggcgcacga gagcaacgag atgcagccga cgctcgccat 1321
cagccatgcc ggggtcagcg tggtcatggc ccagacccag ccgcgccggg aaaagcgctg 1381
gagcgaatgg gccagcggca aggtgttgtg cctgctcgac ccgctggacg gggtctacaa 1441
ctacctcgcc cagcaacgct gcaacctcga cgatacctgg gaaggcaaga tctaccgggt 1501
gctcgccggc aacccggcga agcatgacct ggacatcaaa cccacggtca tcagtcatcg 1561
cctgcacttt cccgagggcg gcagcctggc cgcgctgacc gcgcaccagg cttgccacct 1621
gccgctggag actttcaccc gtcatcgcca gccgcgcggc tgggaacaac tggagcagtg 1681
cggctatccg gtgcagcggc tggtcgccct ctacctggcg gcgcggctgt cgtggaacca 1741
ggtcgaccag gtgatccgca acgccctggc cagccccggc agcggcggcg acctgggcga 1801
agcgatccgc gagcagccgg agcaggcccg tctggccctg accctggccg ccgccgagag 1861
cgagcgcttc gtccggcagg gcaccggcaa cgacgaggcc ggcgcggcca acgccgacgt 1921
ggtgagcctg acctgcccgg tcgccgccgg tgaatgcgcg ggcccggcgg acagcggcga 1981
cgccctgctg gagcgcaact atcccactgg cgcggagttc ctcggcgacg gcggcgacgt 2041
cagcttcagc acccgcggca cgcagaactg gacggtggag cggctgctcc aggcgcaccg 2101
ccaactggag gagcgcggct atgtgttcgt cggctaccac ggcaccttcc tcgaagcggc 2161
gcaaagcatc gtcttcggcg gggtgcgcgc gcgcagccag gacctcgacg cgatctggcg 2221
cggtttctat atcgccggcg atccggcgct ggcctacggc tacgcccagg accaggaacc 2281
cgacgcacgc ggccggatcc gcaacggtgc cctgctgcgg gtctatgtgc cgcgctcgag 2341
cctgccgggc ttctaccgca ccagcctgac cctggccgcg ccggaggcgg cgggcgaggt 2401
cgaacggctg atcggccatc cgctgccgct gcgcctggac gccatcaccg gccccgagga 2461
ggaaggcggg cgcctggaga ccattctcgg ctggccgctg gccgagcgca ccgtggtgat 2521
tccctcggcg atccccaccg acccgcgcaa cgtcggcggc gacctcgacc cgtccagcat 2581
ccccgacaag gaacaggcga tcagcgccct gccggactac gccagccagc ccggcaaacc 2641
gccgcgcgag gacctgaagt aactgccgcg accggccggc tcccttcgca ggagccggcc 2701
ttctcggggc ctggccatac atcaggtttt cctgatgcca gcccaatcga atatgaattc 2761
```

GENBANK Accession No. K01397 M23348.

The encoded amino acid sequence of ETA (SEQ ID NO:16) is

```
MHLIPHWIPL VASLGLLAGG SSASAAEEAF DLWNECAKAC VLDLKDGVRS SRMSVDPAIA
DTNGQGVLHY SMVLEGGNDA LKLAIDNALS ITSDGLTIRL EGGVEPNKPV RYSYTRQARG
SWSLNWLVPI GHEKPSNIKV FIHELNAGNQ LSHMSPIYTI EMGDELLAKL ARDATFFVRA
HESNEMQPTL AISHAGVSVV MAQTQPRREK RWSEWASGKV LCLLDPLDGV YNYLAQQRCN
LDDTWEGKIY RVLAGNPAKH DLDIKPTVIS HRLHFPEGGS LAALTAHQAC HLPLETFTRH
RQPRGWEQLE QCGYPVQRLV ALYLAARLSW NQVDQVIRNA LASPGSGGDL GEAIREQPEQ
ARLALTLAAA ESERFVRQGT GNDEAGAANA DVVSLTCPVA AGECAGPADS GDALLERNYP
TGAEFLGDGG DVSFSTRGTQ NWTVERLLQA HRQLEERGYV FVGYHGTFLE AAQSIVFGGV
RARSQDLDAI WRGFYIAGDP ALAYGYAQDQ EPDARGRIRN GALLRVYVPR SSLPGFYRTS
LTLAAPEAAG EVERLIGHPL PLRLDAITGP EEEGGRLETI LGWPLAERTV VIPSAIPTDP
RNVGGDLDPS SIPDKEQAIS ALPDYASQPG KPPREDLK
```

GENBANK Accession No. K01397 M23348; residues 1-25 = signal peptide; start of mature peptide is the underscored "A"; the ETA translocation domain, which is a useful IPP according to this invention, spans residues 247-417 (underscored, bolded) of SEQ ID NO_, above.

The sequence of the Flt3 Ligand (FL) extracellular domain is shown below :

(nucleic acid is SEQ ID NO:17; amino acids are SEQ ID NO:18)

```
1/1                                                    31/11
atg aca gtg ctg gcg cca gcc tgg agc cca aat tcc tcc ctg ttg ctg ctg ttg ctg ctg
Met thr val leu ala pro ala trp ser pro asn ser ser leu leu leu leu leu leu leu
```

```
61/21                                                91/31
ctg agt cct tgc ctg cgg ggg aca cct gac tgt tac ttc agc cac agt ccc atc tcc tcc
leu ser pro cys leu arg gly thr pro asp cys tyr phe ser his ser pro ile ser ser
121/41                                               151/51
aac ttc aaa gtg aag ttt aga gag ttg act gac cac ctg ctt aaa gat tac cca gtc act
asn phe lys val lys phe arg glu leu thr asp his leu leu lys asp tyr pro val thr
181/61                                               211/71
gtg gcc gtc aat ctt cag gac gag aag cac tgc aag gcc ttg tgg agc ctc ttc cta gcc
val ala val asn leu gln asp glu lys his cys lys ala leu trp ser leu phe leu ala
241/81                                               271/91
cag cgc tgg ata gag caa ctg aag act gtg gca ggg tct aag atg caa acg ctt ctg gag
gln arg trp ile glu gln leu lys thr val ala gly ser lys met gln thr leu leu glu
301/101                                              331/111
gac gtc aac acc gag ata cat ttt gtc acc tca tgt acc ttc cag ccc cta cca gaa tgt
asp val asn thr glu ile his phe val thr ser cys thr phe gln pro leu pro glu cys
361/121                                              391/131
ctg cga ttc gtc cag acc aac atc tcc cac ctc ctg aag gac acc tgc aca cag ctg ctt
leu arg phe val gln thr asn ile ser his leu leu lys asp thr cys thr gln leu leu
421/141                                              451/151
gct ctg aag ccc tgt atc ggg aag gcc tgc cag aat ttc tct cgg tgc ctg gag gtg cag
ala leu lys pro cys ile gly lys ala cys gln asn phe ser arg cys leu glu val gln
481/161                                              511/171
tgc cag ccg gac tcc tcc acc ctg ctg ccc cca agg agt ccc ata gcc cta gaa gcc acg
cys gln pro asp ser ser thr leu leu pro pro arg ser pro ile ala leu glu ala thr
541/181
gag ctc cca gag cct cgg ccc agg cag
glu leu pro glu pro arg pro arg gln
```

A fusion polypeptide FL-E7 is shown below:

(nucleic acid is SEQ ID NO:19; amino acids are SEQ ID NO:20). The N-terminal sequence is FL, followed by E7 (underscored, nucleic acids capitalized)

```
1/1                                                  31/11
atg aca gtg ctg gcg cca gcc tgg agc cca aat tcc tcc ctg ttg ctg ctg ttg ctg ctg
Met thr val leu ala pro ala trp ser pro asn ser ser leu leu leu leu leu leu leu
61/21                                                91/31
ctg agt cct tgc ctg cgg ggg aca cct gac tgt tac ttc agc cac agt ccc atc tcc tcc
leu ser pro cys leu arg gly thr pro asp cys tyr phe ser his ser pro ile ser ser
121/41                                               151/51
aac ttc aaa gtg aag ttt aga gag ttg act gac cac ctg ctt aaa gat tac cca gtc act
asn phe lys val lys phe arg glu leu thr asp his leu leu lys asp tyr pro val thr
181/61                                               211/71
gtg gcc gtc aat ctt cag gac gag aag cac tgc aag gcc ttg tgg agc ctc ttc cta gcc
val ala val asn leu gln asp glu lys his cys lys ala leu trp ser leu phe leu ala
241/81                                               271/91
cag cgc tgg ata gag caa ctg aag act gtg gca ggg tct aag atg caa acg ctt ctg gag
gln arg trp ile glu gln leu lys thr val ala gly ser lys met gln thr leu leu glu
301/101                                              331/111
gac gtc aac acc gag ata cat ttt gtc acc tca tgt acc ttc cag ccc cta cca gaa tgt
asp val asn thr glu ile his phe val thr ser cys thr phe gln pro leu pro glu cys
361/121                                              391/131
ctg cga ttc gtc cag acc aac atc tcc cac ctc ctg aag gac acc tgc aca cag ctg ctt
leu arg phe val gln thr asn ile ser his leu leu lys asp thr cys thr gln leu leu
421/141                                              451/151
gct ctg aag ccc tgt atc ggg aag gcc tgc cag aat ttc tct cgg tgc ctg gag gtg cag
ala leu lys pro cys ile gly lys ala cys gln asn phe ser arg cys leu glu val gln
481/161                                              511/171
tgc cag ccg gac tcc tcc acc ctg ctg ccc cca agg agt ccc ata gcc cta gaa gcc acg
cys gln pro asp ser ser thr leu leu pro pro arg ser pro ile ala leu glu ala thr
541/181                                              571/191
gag ctc cca gag cct cgg ccc agg cag gaa ttc ATG CAT GGA GAT ACA CCT ACA TTG CAT
glu leu pro glu pro arg pro arg gln glu phe met his gly asp thr pro thr leu his
601/201                                              631/211
GAA TAT ATG TTA GAT TTG CAA CCA GAG ACA ACT GAT CTC TAC TGT TAT GAG CAA TTA AAT
glu tyr met leu asp leu gln pro glu thr thr asp leu tyr cys tyr glu gln leu asn
661/221                                              691/231
GAC AGC TCA GAG GAG GAG GAT GAA ATA GAT GGT CCA GCT GGA CAA GCA GAA CCG GAC AGA
asp ser ser glu glu glu asp glu ile asp gly pro ala gly gln ala glu pro asp arg
```

```
721/241                                          751/251
GCC CAT TAC AAT ATT GTA ACC TTT TGT TGC AAG TGT GAC TCT ACG CTT CGG TTG TGC GTA
ala his tyr asn ile val thr phe cys cys lys cys asp ser thr leu arg leu cys val
781/261                                          811/271
CAA AGC ACA CAC GTA GAC ATT CGT ACT TTG GAA GAC CTG TTA ATG GGC ACA CTA GGA ATT
gln ser thr his val asp ile arg thr leu glu asp leu leu met gly thr leu gly ile
841/281
GTG TGC CCC ATC TGT TCT CAA GGA TCC
val cys pro ile cys ser gln gly ser
```

The sequences of CRT, including human CRT, are well known in the art (McCauliffe (1990) J. Clin. Invest. 86:332-335; Burns (1994) Nature 367:476-480; Coppolino (1998) Int. J. Biochem. Cell Biol. 30:553-558). The nucleic acid sequence appears as GenBank Accession No. NM 004343 and is SEQ ID NO:21.

```
1    gtccgtactg cagagccgct gccggagggt cgttttaaag ggccgcgttg ccgccccctc
61   ggcccgccat gctgctatcc gtgccgctgc tgctcggcct cctcggcctg gccgtcgccg
121  agcccgccgt ctacttcaag gagcagtttc tggacggaga cgggtggact tcccgctgga
181  tcgaatccaa acacaagtca gattttggca aattcgttct cagttccggc aagttctacg
241  gtgacgagga gaaagataaa ggtttgcaga caagccagga tgcacgcttt tatgctctgt
301  cggccagttt cgagcctttc agcaacaaag gccagacgct ggtggtgcag ttcacggtga
361  aacatgagca gaacatcgac tgtgggggcg gctatgtgaa gctgtttcct aatagtttgg
421  accagacaga catgcacgga gactcagaat acaacatcat gtttggtccc gacatctgtg
481  gccctggcac caagaaggtt catgtcatct tcaactacaa gggcaagaac gtgctgatca
541  acaaggacat ccgttgcaag gatgatgagt ttacacacct gtacacactg attgtgcggc
601  cagacaacac ctatgaggtg aagattgaca acagccaggt ggagtccggc tccttggaag
661  acgattggga cttcctgcca cccaagaaga taaaggatcc tgatgcttca aaaccggaag
721  actgggatga gcgggccaag atcgatgatc ccacagactc caagcctgag gactgggaca
781  agcccgagca tatccctgac cctgatgcta agaagcccga ggactgggat gaagagatgg
841  acggagagtg ggaaccccca gtgattcaga accctgagta caagggtgag tggaagcccc
901  ggcagatcga caacccagat tacaagggca cttggatcca cccagaaatt gacaaccccg
961  agtattctcc cgatcccagt atctatgcct atgataactt tggcgtgctg ggcctggacc
1021 tctggcaggt caagtctggc accatctttg acaacttcct catcaccaac gatgaggcat
1081 acgctgagga gtttggcaac gagacgtggg gcgtaacaaa ggcagcagag aaacaaatga
1141 aggacaaaca ggacgaggag cagaggctta aggaggagga agaagacaag aaacgcaaag
1201 aggaggagga ggcagaggac aaggaggatg atgaggacaa agatgaggat gaggaggatg
1261 aggaggacaa ggaggaagat gaggaggaag atgtccccgg ccaggccaag gacgagctgt
1321 agagaggcct gcctccaggg ctggactgag gcctgagcgc tcctgccgca gagcttgccg
1381 cgccaaataa tgtctctgtg agactcgaga actttcattt ttttccaggc tggttcggat
1441 ttggggtgga ttttggtttt gttcccctcc tccactctcc cccacccccc ccccgccctt
1501 ttttttttt ttttaaact ggtattttat cctttgattc tccttcagcc ctcacccctg
1561 gttctcatct ttcttgatca acatcttttc ttgcctctgt gccccttctc tcatctctta
1621 gctcccctcc aacctggggg gcagtggtgt ggagaagcca caggcctgag atttcatctg
1681 ctctccttcc tggagcccag aggagggcag cagaaggggg tggtgtctcc aaccccccag
1741 cactgaggaa gaacggggct cttctcattt caccctccc tttctcccct gcccccagga
1801 ctgggccact tctgggtggg gcagtgggtc ccagattggc tcacactgag aatgtaagaa
1861 ctacaaacaa aatttctatt aaattaaatt ttgtgtctc                    1899
```

Human CRT protein (GenBank Accession No. NM 004343), (SEQ ID NO:22) is shown below:

```
1    MLLSVPLLLG LLGLAVAEPA VYFKEQFLDG DGWTSRWIES KHKSDFGKFV LSSGKFYGDE
61   EKDKGLQTSQ DARFYALSAS FEPFSNKGQT LVVQFTVKHE QNIDCGGGYV KLFPNSLDQT
121  DMHGDSEYNI MFGPDICGPG TKKVHVIFNY KGKNVLINKD IRCKDDEFTH LYTLIVRPDN
181  TYEVKIDNSQ VESGSLEDDW DFLPPKKIKD PDASKPEDWD ERAKIDDPTD SKPEDWDKPE
241  HIPDPDAKKP EDWDEEMDGE WEPPVIQNPE YKGEWKPRQI DNPDYKGTWI HPEIDNPEYS
301  PDPSIYAYDN FGVLGLDLWQ VKSGTIFDNF LITNDEAYAE EFGNETWGVT KAAEKQMKDK
361  QDEEQRLKEE EEDKKRKEEE EAEDKEDDED KDEDEEDEED KEEDEEEDVP GQAKDEL 417
```

ANTIGENS

In one embodiment, the antigen of the present invention against which immunity is desired and which may be as short as an MHC class I-binding peptide epitope, is derived from a pathogen, *e.g.*, it comprises a peptide expressed by a pathogen. The pathogen can be a virus, such as, *e.g.*, a papilloma virus, a herpesvirus, a retrovirus (*e.g.*, an immunodeficiency virus, such as HIV-1), an adenovirus, and the like. The papilloma virus can be a human papilloma virus; for example, the antigen (*e.g.*, the Class I-binding peptide) can be derived from an HPV-16 E7 polypeptide. In one embodiment, the HPV-16 E7 polypeptide is substantially non-oncogenic, *i.e.*, it does not bind retinoblastoma polypeptide (pRB) or binds pRB with such low affinity that the HPV-16 E7 polypeptide is effectively non-oncogenic when expressed or delivered *in vivo*.

In alternative embodiments, the pathogen is a bacteria, such as*Bordetella pertussis*; *Ehrlichia chaffeensis*; *Staphylococcus aureus*; *Toxoplasma gondii*; *Legionella pneumophila*; *Brucella suis*; *Salmonella enterica*; *Mycobacterium avium*; *Mycobacterium tuberculosis*; *Listeria monocytogenes*; *Chlamydia trachomatis*; *Chlamydia pneumoniae*; *Rickettsia rickettsii*; or, a fungi, such as, *e.g.*, *Paracoccidioides brasiliensis*; or other pathogen, *e.g.*, *Plasmodium falciparum*.

In another embodiment, the MHC class I-binding peptide epitope is derived from a tumor cell. The tumor cell-derived peptide epitope can comprise a tumor associated antigen, *e.g.*, a tumor specific antigen, such as, *e.g.*, a HER-2/neu antigen.

Generating and Manipulating of Nucleic Acids

The methods of the invention provide for the administration of nucleic acid vectors encoding a fusion protein between an antigen, preferably a MHC Class I epitope binding polypeptide or peptide, used to an IPP, as has been described above.

Recombinant IPP- containing fusion proteins can be synthesized *in vitro* or *in vivo*. Nucleic acids encoding these compositions can be prepared in in the form of "naked DNA" or they can be incorporated in plasmids, vectors, recombinant viruses (*e.g.*, "replicons") and the like. The present invention is directed ot one class of vectors, replication defective alphavirus vectors, prefereably Sinbis virus, for *in vivo* or *ex vivo* administration. Nucleic acids and vectors of the invention can be made and expressed *in vitro* or *in vivo*, a variety of means of making and expressing these genes and vectors can be used. One of skill will recognize that desired expression can be obtained by modulating the activity of the nucleic acids (*e.g.*, promoters) within vectors used to practice the invention. Any of the known methods described for increasing or decreasing expression or activity, or tissue specificity, of genes can be used for this invention. The invention can be practiced in conjunction with any method or protocol known in the art, which are well described in the scientific and patent literature.

GENERAL RECOMBINANT DNA METHODS

Basic texts disclosing general methods of molecular biology, all of which are incorporated by reference, include: Sambrook, J *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989; Ausubel, FM *et al. Current Protocols in Molecular Biology*, Vol. 2, Wiley-Interscience, New York, (current edition); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); Glover, DM, ed, *DNA Cloning: A Practical Approach*, vol. I & II, IRL Press, 1985; Albers, B. *et al.*, *Molecular Biology of the Cell*, 2nd Ed., Garland Publishing, Inc., New York, NY (1989); Watson, JD *et al.*, *Recombinant DNA*, 2nd Ed., Scientific American Books, New York, 1992; and Old, RW *et al.*, *Principles of Gene Manipulation: An Introduction to Genetic Engineering*, 2nd Ed., University of California Press, Berkeley, CA (1981).

Unless otherwise indicated, a particular nucleic acid sequence is intended to encompasses conservative substitution variants thereof *(e.g.,* degenerate codon substitutions) and a complementary sequence. The term "nucleic acid" is synonymous with "polynucleotide" and is intended to include a gene, a cDNA molecule, an mRNA molecule, as well as a fragment of any of these such as an oligonucleotide, and further, equivalents thereof (explained more fully below). Sizes of nucleic acids are stated either as kilobases (kb) or base pairs (bp). These are estimates derived from agarose or polyacrylamide gel electrophoresis (PAGE), from nucleic acid sequences which are determined by the user or published. Protein size is stated as molecular mass in kilodaltons (kDa) or as length (number of amino acid residues). Protein size is estimated from PAGE, from sequencing, from presumptive amino acid sequences based on the coding nucleic acid sequence or from published amino acid sequences.

Specifically, cDNA molecules encoding the amino acid sequence corresponding to the fusion polypeptide of the present invention or fragments or derivatives thereof can be synthesized by the polymerase chain reaction (PCR) (see, for example, U.S. 4,683,202) using primers derived the sequence of the protein disclosed herein. These cDNA sequences can then be assembled into a eukaryotic or prokaryotic expression vector and the resulting vector can be used to direct the synthesis of the fusion polypeptide or its fragment or derivative by appropriate host cells, for example COS or CHO cells.

This invention includes isolated nucleic acids having a nucleotide sequence encoding the novel fusion polypeptides that comprise a spreading protein and an antigen, fragments thereof or equivalents thereof. The term nucleic acid as used herein is intended to include such fragments or equivalents. The nucleic acid sequences of this invention can be DNA or RNA.

A cDNA nucleotide sequence the fusion polypeptide can be obtained by isolating total mRNA from an appropriate cell line. Double stranded cDNA is prepared from total mRNA. cDNA can be inserted into a suitable plasmid, bacteriophage or viral vector using any one of a number of known techniques.

In reference to a nucleotide sequence, the term "equivalent" is intended to include sequences encoding structurally homologous and/or a functionally equivalent proteins. For example, a natural polymorphism of a sequence encoding an IPP such as viral VP22 spreading protein or CRT, or the like, (especially at the third base of a codon) may be manifest as "silent" mutations which do not change the amino acid sequence. Furthermore, there may be one or more naturally occurring isoforms or related, immunologically cross-reactive family members of the an IPP such as VP22. Such isoforms or family members are defined as proteins that share function amino acid sequence similarity to the reference protein.

Fragment of Nucleic Acid

A fragment of the nucleic acid sequence is defined as a nucleotide sequence having fewer nucleotides than the nucleotide sequence encoding the full length IPP, antigenic polypeptide or the fusion thereof.. This invention includes such nucleic acid fragments that encode polypeptides which retain (1) the ability of the fusion polypeptide to induce increases in frequency or reactivity of T cells, preferably CD8+ T cells, that are specific for the antigen portion of the fusion polypeptide.

For example, a nucleic acid fragment as intended herein encodes a VP22 or HSP70 or CRT or FL or other type of IPP that retains the ability to improve the immunogenicity of an antigen when administered as a fusion polypeptide with an antigenic polypeptide or peptide.

Generally, the nucleic acid sequence encoding a fragment of an IPP polypeptide comprises nucleotides from the sequence encoding the mature protein (*i.e.*, excluding signal peptide sequences).

Nucleic acid sequences of this invention may also include linker sequences, natural or modified restriction endonuclease sites and other sequences that are useful for manipulations related to cloning, expression or purification of encoded protein or fragments. These and other modifications of nucleic acid sequences are described herein or are well-known in the art.

The techniques for assembling and expressing DNA coding sequences for IPPs such as spreading proteins, proteins or ER-such as VP22 and antigenic polypeptides such as synthesis of oligonucleotides, PCR, transforming cells, constructing vectors, expression systems, and the like

are well-established in the art. Those of ordinary skill are familiar with the standard resource materials for specific conditions and procedures.

EXPRESSION VECTORS AND HOST CELLS

This invention includes an expression vector comprising a nucleic acid sequence encoding a spreading protein/antigen fusion polypeptide operably linked to at least one regulatory sequence. "Operably linked" means that the coding sequence is linked to a regulatory sequence in a manner that allows expression of the coding sequence. Known regulatory sequences are selected to direct expression of the desired protein in an appropriate host cell. Accordingly, the term "regulatory sequence" includes promoters, enhancers and other expression control elements. Such regulatory sequences are described in, for example, Goeddel, *Gene Expression Technology. Methods in Enzymology,* vol. 185, Academic Press, San Diego, Calif. (1990)).

Those skilled in the art appreciate that the particular design of an expression vector of this invention depends on considerations such as the host cell to be transfected and/or the type of protein to be expressed.

The present expression vectors comprise the full range of nucleic acid molecules encoding the various embodiments of the fusion polypeptide and its functional derivatives (defined herein) including polypeptide fragments, variants, *etc.*

Such expression vectors are used to transfect host cells for expression of the DNA and production of the encoded proteins which include fusion proteins or peptides. It will be understood that a genetically modified cell expressing the fusion polypeptide may transiently express the exogenous DNA for a time sufficient for the cell to be useful for its stated purpose.

The present in invention provides methods for producing the fusion polypeptides, fragments and derivatives. For example, a host cell transfected with a nucleic acid vector that encodes the fusion polypeptide is cultured under appropriate conditions to allow expression of the polypeptide.

Host cells may also be transfected with one or more expression vectors that singly or in combination comprise DNA encoding at least a portion of the fusion polypeptide and DNA encoding at least a portion of a second protein, so that the host cells produce yet further fusion polypeptides that include both the portions.

A culture typically includes host cells, appropriate growth media and other byproducts. Suitable culture media are well known in the art. The fusion polypeptide can be isolated from medium or cell lysates using conventional techniques for purifying proteins and peptides, including ammonium sulfate precipitation, fractionation column chromatography (e.g. ion exchange, gel filtration, affinity chromatography, etc.) and/or electrophoresis (see generally, "Enzyme Purification and Related Techniques", *Meth Enzymol 22*: 233-577 (1971)). Once purified, partially or to homogeneity, the recombinant polypeptides of the invention can be utilized in pharmaceutical compositions as described in more detail herein.

Prokaryotic or eukaryotic host cells transformed or transfected to express the fusion polypeptide or a homologue or functional derivative thereof are within the scope of the invention. For example, the fusion polypeptide may be expressed in bacterial cells such as *E. coli*, insect cells (baculovirus), yeast, or mammalian cells such as Chinese hamster ovary cells (CHO) or human cells. Other suitable host cells may be found in Goeddel, (1990) *supra* or are otherwise known to those skilled in the art.

Expression in eukaryotic cells leads to partial or complete glycosylation and/or formation of relevant inter- or intra-chain disulfide bonds of the recombinant protein.

PCL-generated replication-defective Alpha virus replicons

Vector systems for the expression of heterologous genes have been developed from full-length cDNA clones of three members of the alphavirus genus, Sindbis virus ("SIN"), Semliki Forest virus (SFV), and Venezuelan equine encephalitis virus (VEE) (Xiong *et al.*, 1989; Huang, HV *et al.*, 1989, *Virus Genes 3*:85–91; Liljestrom, P *et al.*, , 1991, *BioTechnology 9*:1356–1361; Bredenbeek, PJ *et al.*, 1993, *J. Virol. 67*:6439–6446; Zhou, X *et al.*, 1994, *Vaccine 12*:1510–1514; Davis *et al.*, 1996, *J. Virol. 70*:3781–3787; Dryga, SA *et al.*, 1996, (Russian). *Vopr. Virusol.* 3:100-104; reviewed in Frolov *et al.*, *supra*. The systems are of two general types: ''double promoter'' vectors and ''replicon'' vectors.

In alphavirus replicon vectors, the viral structural protein genes are deleted and replaced by a heterologous gene under the control of the 26S promoter. The structural genes are provided in trans from a helper construct(s) consisting of a partial clone of the viral genome which is missing all or part of the genes for the nonstructural proteins, including the putative cis-acting RNA packaging signals (Weiss, B *et al.*, 1989, *J. Virol. 63*:5310–5318; Weiss, B. *et al.*, 1994, *Nucleic Acids Res. 22*:780–786). Both the replicon and the helper RNAs retain the cis-acting

terminal sequences required for genome replication and the promoter for transcription of the subgenomic mRNA. The replicon RNA supplies the nonstructural proteins in trans for the expression of the helper RNA. However, only the replicon RNA retains the packaging signal(s), and it is packaged into replicon particles by the viral structural proteins provided in trans by the helper. Infection of cells by these replicon particles results in amplification of replicon RNA and expression of the heterologous gene, but there is no further spread to other cells.

Alphavirus replicon vectors have been used to vaccinate against microbial pathogens (Zhou, X *et al.*, 1995, *Proc. Natl. Acad. Sci. USA 92*:3009–3013; Mossman, SP *et al.*, 1996, *J. Virol. 70*:1953–1960) and offer several potential advantages as delivery systems. As noted above, replicons typically express heterologous genes to high levels, permittin a relatively low dose of replicon particles to produce a large dose of immunogen *in vivo*. Because they lack a complete complement of viral genes, after replication and expression of the heterologous gene in the cells initially infected, no additional infectious particles would be produced to spread to other tissues. This property contributes significantly to the inherent safety of alphavirus replicon vectors.

In addition, the self-limiting nature of the replicon particle infection and the lack of structural protein expression should minimize the induction of an immune response to the vector, allowing the sequential use of these vectors for immunization of the same individual with immunogens of different pathogens.

As described in Pushko *et al.*, 1997, VEE was the only alphavirus for which a live, attenuated vaccine strain (TC-83) has been developed for veterinary and human use (Jahrling, PB *et al.*, 1984, *J. Clin. Microbiol. 19:*429–431; Kinney, R *et al.*, 1993, *J. Virol. 67*:1269–1277). Newer live, attenuated VEE vaccines with improvements over TC-83, were been developed from a full-length cDNA clone (Davis, NL *et al.*, 1989, *Virology 171*:189–204), by the introduction of multiple attenuating mutations into the structural genes. Thus, vectors derived from such vaccine strains are inherently safer than those derived from wild-type virus. Several of these strains, including V3014, replicate in lymphoid tissue without causing disease (Pushko *et al.*, *supra*). In the context of a vector, the VEE glycoproteins will preferentially target heterologous gene expression to lymphoid tissues (Davis, NL *et al.*, 1996, *J. Virol. 70*:3781–3787; Caley, IJ *et al.*, 1997, *J. Virol. 71*:3031–3038), which would be expected to increase immunogenicity of the heterologous gene product.

Two disclosed strategies for improving the safety and efficacy of a VEE-based replicon vector (Pushko *et al.*, *supra*) either (1) included previously defined attenuating mutations in the replicon and/or its helper so that any viable recombinant virus is could not initiate a virulent infection; or (2) used a bipartite helper to supply structural proteins for packaging of the replicon into particles, thus requiring at least two recombination events for the generation of viable virus. This strategy greatly reduces the probability that a viable recombinant VEE virus would be generated during packaging. Using a VEE replicon particle vaccine prepared in this manner, Pushko and colleagues induced potent protective immunity to a heterologous mucosal pathogen in naive animals and have achieved an equally high level, protective response in animals previously immunized with VEE replicon particle-expressing genes from another pathogen.

The utility of the alphavirus replicon expression systems has been markedly improved by development of a series of defective helper RNAs that allow efficient packaging of RNA replicons (Liljestrom *et al.*, *supra*; Bredenbeek *et al.*, *supra*). Defective-helper RNAs (DHRNAs) are designed to contain the cis-acting sequences required for replication as well as the subgenomic RNA promoter driving expression of the structural protein genes. Packaging of SIN replicons is achieved by efficient cotransfection of BHK cells with both RNAs by electroporation (Liljestrom *et al.*, 1991, *supra*) (See also, Frolov *et al.*, 1996, *supra*; Fig. 3). Replicase/transcriptase functions supplied by the vector RNA lead not only to its own amplification but also act in trans to allow replication and transcription of the helper RNA. This results in synthesis of structural proteins that can package the replicon with $>10^8$ infectious particles per ml ($5x10^9$ infectious particles per electroporation) being produced after only 16–24 h. Such stocks can be used without further phenotypic selection to infect cells for expression studies or high-level protein production. According to Frolov *et al.*,*supra*, it should be possible to package replicons containing at least 5 kb of heterologous sequence.

A spectrum of DHRNAs have been characterized that differ in their ability to be packaged. Some DHRNAs that allow packaging of the replicon as well as themselves are useful under conditions where extensive amplification by passaging is advantageous. Other DHRNAs allow efficient packaging of replicons but are packaged very poorly themselves (Frolov *et al.*, *supra*; Liljestrom *et al.*, *supra*; Bredenbeek *et al.*, *supra*; Geigenmuller-Gnirke *et al.*, *supra*). These latter helpers are useful when expression of viral structural proteins and virus spread are not desired.

One approach to minimize the possibility of recombination between replicon and helper RNAs to produce wild-type virus is to use two DHRNAs, one that expresses the capsid protein and a second that expresses the viral glycoproteins (Frolov *et al.*, *supra*). The capsid protein, expressed independently, accumulates at high levels, but to achieve similar levels of viral glycoprotein expression retention of the 5' terminus of the capsid protein mRNA, which acts as a translational enhancer, is required. Deletions in the capsid protein gene that preserve both the 5' terminus (the enhancer region) and the 3' half (the sequences that code for the autoprotease activity) but eliminate the region that binds RNA, produce high levels of glycoprotein expression from a second DHRNA. Capsid protein genes from heterologous alphaviruses can also be used to enhance translation of the glycoproteins and should further reduce the probability of wild-type virus generation via recombination.

In addition to packaging of alphavirus RNA replicons by cotransfection with DHRNAs, continuous packaging cell lines have been developed that express a DHRNA under the control of a nuclear promoter. Such cells may be useful for rescuing transfected RNA replicons, titering packaged replicons, and production of large quantities of packaged replicon stocks by low-multiplicity passage.

Variants of the prototype alphavirus, SIN, with differential abilities to infect human dendritic cells were described by Gardner JP *et al.*, 2000, *J Virol 74*:11849-11857. The genetic determinant for human DC tropism maps to a single amino acid substitution at residue 160 of the envelope glycoprotein E2. Packaging of SIN replicon vectors with the E2 glycoprotein from a DC-tropic variant confers a similar ability to efficiently infect immature human DC, whhich are induced to undergo rapid activation and maturation. The SIN replicon particles infected skin-resident mouse DC *in vivo*, which subsequently migrated to the draining lymph nodes and upregulated cell surface expression of MHC and costimulatory molecules. SIN replicon particles encoding HIV 1 p55(Gag) elicited potent specific T cell responses *in vitro* and *in vivo* , demonstrating that infected DC maintained their ability to process and present replicon-encoded antigen. Human and mouse DC were differentially infected by selected SIN variants, suggesting differences in receptor expression between human and murine DC.

Thus, the present invention provides a dual approach to enhancing the potency of nucleic acid vaccines. On the one hand, the present nucleic acid constructs are designed to target MHC class I processing pathways, to target DC's, to stimulate DC maturation, activation, *etc.*, as

described. However, independently, the vector system provides a potential of using a directed approach to generate ialphavirus vaccine vectors that target and activate APCs.

Although preferred vectors and packaging cells are described in the Examples, other examples of alphavirus replicons as expression vectors are noted above are well-known in the art, as are corresponding packaging cells that permit their production in relatively high quantities.

For certain fusion expression vectors, a proteolytic cleavage site may be introduced at the junction of the reporter group and the target protein to enable separation of the target protein from the reporter group subsequent to purification of the fusion protein. Proteolytic enzymes for such cleavage and their recognition sequences include Factor Xa, thrombin and enterokinase.

One embodiment of this invention is a transfected cell which expresses novel fusion polypeptide.

Vector Construction

In construction of suitable vectors containing the desired coding and control sequences in the process of arriving at the PCL-generated replication-defective Sinbis virus replicons of the present invention, standard ligation and restriction techniques which are well understood in the art are employed. Isolated plasmids, DNA sequences, or synthesized oligonucleotides are cleaved, tailored, and re-ligated in the form desired.

The DNA sequences which are used for the present constructs are available from a number of sources. Backbone vectors and control systems are generally found on available "host" vectors which are used for the bulk of the sequences in construction. For the pertinent coding sequence, initial construction may be, and usually is, a matter of retrieving the appropriate sequences from cDNA or genomic DNA libraries. However, once the sequence is disclosed it is possible to synthesize the entire gene sequence *in vitro* starting from the individual nucleotide derivatives. The entire gene sequence for genes of sizeable length, *e.g.*, 500-1000 bp may be prepared by synthesizing individual overlapping complementary oligonucleotides and filling in single stranded nonoverlapping portions using DNA polymerase in the presence of the deoxyribonucleotide triphosphates. This approach has been used successfully in the construction of several genes of known sequence. See, for example, Edge, M. D., *Nature* (1981) 292:756; Nambair, K. P., *et al.*, *Science* (1984) 223:1299; and Jay, E., *J Biol Chem* (1984) 259:6311.

Synthetic oligonucleotides are prepared by either the phosphotriester method as described by references cited above or the phosphoramidite method as described by Beaucage, S. L., and Caruthers, M. H., *Tet Lett* (1981) 22:1859; and Matteucci, M. D., and Caruthers, M. H., *J Am Chem Soc* (1981) 103:3185 and can be prepared using commercially available automated oligonucleotide synthesizers. Kinase treatment of single strands prior to annealing or for labeling is achieved using an excess, e.g., about 10 units of polynucleotide kinase to 1 nmole substrate in the presence of 50 mM Tris, pH 7.6, 10 mM $MgCl_2$, 5 mM dithiothreitol, 1-2 mM ATP, 1.7 pmoles γ-^{32}P-ATP (2.9 mCi/mmole), 0.1 mM spermidine, 0.1 mM EDTA.

Once the components of the desired vectors are thus available, they can be excised and ligated using standard restriction and ligation procedures. Site-specific DNA cleavage is performed by treating with the suitable restriction enzyme (or enzymes) under conditions which are generally understood in the art, and the particulars of which are specified by the manufacturer of these commercially available restriction enzymes. See, *e.g.*, New England Biolabs, Product Catalog. In general, about 1 mg of plasmid or DNA sequence is cleaved by one unit of enzyme in about 20 ml of buffer solution; in the examples herein, typically, an excess of restriction enzyme is used to insure complete digestion of the DNA substrate. Incubation times of about one hour to two hours at about 37°C. are workable, although variations can be tolerated. After each incubation, protein is removed by extraction with phenol/chloroform, and may be followed by ether extraction, and the nucleic acid recovered from aqueous fractions by precipitation with ethanol. If desired, size separation of the cleaved fragments may be performed by polyacrylamide gel or agarose gel electrophoresis using standard techniques. A general description of size separations is found in *Methods in Enzymology* (1980) 65:499-560.

Restriction cleaved fragments may be blunt ended by treating with the large fragment of *E. coli* DNA polymerase I (Klenow) in the presence of the four deoxynucleotide triphosphates (dNTPs) using incubation times of about 15 to 25 min at 20° to 25° C. in 50 mM Tris pH 7.6, 50 mM NaCl, 6 mM $MgCl_2$, 6 mM DTT and 0.1-1.0 mM dNTPs. The Klenow fragment fills in at 5′ single-stranded overhangs but chews back protruding 3′ single strands, even though the four dNTPs are present. If desired, selective repair can be performed by supplying only one of the, or selected, dNTPs within the limitations dictated by the nature of the overhang. After treatment with Klenow, the mixture is extracted with phenol/chloroform and ethanol precipitated.

Treatment under appropriate conditions with S1 nuclease or BAL-31 results in hydrolysis of any single-stranded portion.

Ligations are typically performed in 15-50 ml volumes under the following standard conditions and temperatures: for example, 20 mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 10 mM DTT, 33 μg/ml BSA, 10-50mM NaCl, and either 40 μM ATP, 0.01-0.02 (Weiss) units T4 DNA ligase at 0° C. (for "sticky end" ligation) or 1mM ATP, 0.3-0.6 (Weiss) units T4 DNA ligase at 14° C. (for "blunt end" ligation). Intermolecular "sticky end" ligations are usually performed at 33-100 μg/ml total DNA concentrations (5-100 nM total end concentration). Intermolecular blunt end ligations are performed at 1 mM total ends concentration.

In vector construction employing "vector fragments", the fragment is commonly treated with bacterial alkaline phosphatase (BAP) or calf intestinal alkaline phosphatase (CIAP) in order to remove the 5' phosphate and prevent self-ligation. Digestions are conducted at pH 8 in approximately 10 mM Tris-HCl, 1 mM EDTA using BAP or CIAP at about 1 unit/mg vector at 60° for about one hour. The preparation is extracted with phenol/chloroform and ethanol precipitated. Alternatively, re-ligation can be prevented in vectors which have been double digested by additional restriction enzyme and separation of the unwanted fragments.

Any of a number of methods are used to introduce mutations into the coding sequence to generate the variants of the invention. These mutations include simple deletions or insertions, systematic deletions, insertions or substitutions of clusters of bases or substitutions of single bases.

For example, modifications of the IPP or the antigenic polypeptide DNA sequence are created by site-directed mutagenesis, a well-known technique for which protocols and reagents are commercially available (Zoller, MJ *et al., Nucleic Acids Res* (1982) 10:6487-6500 and Adelman, JP *et al., DNA* (1983) 2:183-193)). Correct ligations for plasmid construction are confirmed, for example, by first transforming *E. coli* strain MC1061 (Casadaban, M., *et al., J Mol Biol* (1980) 138:179-207) or other suitable host with the ligation mixture. Using conventional methods, transformants are selected based on the presence of the ampicillin-, tetracycline- or other antibiotic resistance gene (or other selectable marker) depending on the mode of plasmid construction. Plasmids are then prepared from the transformants with optional chloramphenicol amplification optionally following chloramphenicol amplification ((Clewell, DB *et al.* , *Proc Natl Acad Sci USA* (1969) 62:1159; Clewell, D. B., *J Bacteriol* (1972) 110:667).

Several mini DNA preps are commonly used. See, *e.g.*,, Holmes, DS, *et al.*, *Anal Biochem* (1981) 114:193-197; Birnboim, HC *et al.* , *Nucleic Acids Res* (1979) 7:1513-1523. The isolated DNA is analyzed by restriction and/or sequenced by the dideoxy nucleotide method of Sanger (*Proc Natl Acad Sci USA* (1977) 74:5463) as further described by Messing, *et al.*, *Nucleic Acids Res* (1981) 9:309, or by the method of Maxam *et al. Methods in Enzymology* (1980) 65:499.

During the process of preparing the nucleic acids of the present invention, vector DNA can be introduced into mammalian cells via conventional techniques such as calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming host cells can be found in Sambrook *et al. supra* and other standard texts.

Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the reporter group and the target protein to enable separation of the target protein from the reporter group subsequent to purification of the fusion protein. Proteolytic enzymes for such cleavage and their recognition sequences include Factor Xa, thrombin and enterokinase.

Typical fusion expression vectors include pGEX (Amrad Corp., Melbourne, Australia), pMAL (New England Biolabs, Beverly, Mass.) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase, maltose E binding protein, or protein A, respectively, to the target recombinant protein.

Inducible non-fusion expression vectors include pTrc (Amann *et al.*, (1988) *Gene* 69: 301-315) and pET 11d (Studier *et al.*, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, Calif. (1990) 60-89). While target gene expression relies on host RNA polymerase transcription from the hybrid trp-lac fusion promoter in pTrc, expression of target genes inserted into pET 11d relies on transcription from the T7 gn10-lacO fusion promoter mediated by coexpressed viral RNA polymerase (T7gn1). Th is viral polymerase is supplied by host strains BL21(DE3) or HMS174(DE3) from a resident λ prophage harboring a T7gn1 under the transcriptional control of the lacUV 5 promoter.

Promoters and Enhancers

A promoter region of a DNA or RNA molecule binds RNA polymerase and promotes the transcription of an "operably linked" nucleic acid sequence. As used herein, a "promoter sequence" is the nucleotide sequence of the promoter which is found on that strand of the DNA or RNA which is transcribed by the RNA polymerase. Two sequences of a nucleic acid

molecule, such as a promoter and a coding sequence, are "operably linked" when they are linked to each other in a manner which permits both sequences to be transcribed onto the same RNA transcript or permits an RNA transcript begun in one sequence to be extended into the second sequence. Thus, two sequences, such as a promoter sequence and a coding sequence of DNA or RNA are operably linked if transcription commencing in the promoter sequence will produce an RNA transcript of the operably linked coding sequence. In order to be "operably linked" it is not necessary that two sequences be immediately adjacent to one another in the linear sequence.

The preferred promoter sequences of the present invention must be operable in mammalian cells and may be either eukaryotic or viral promoters. Although preferred promoters are described in the Examples, other useful promoters and regulatory elements are discussed below. Suitable promoters may be inducible, repressible or constitutive. An example of a constitutive promoter is the viral promoter MSV-LTR, which is efficient and active in a variety of cell types, and, in contrast to most other promoters, has the same enhancing activity in arrested and growing cells. Other preferred viral promoters include that present in the CMV-LTR (from cytomegalovirus) (Bashart, M. *et al.*, *Cell 41*:521 (1985)) or in the RSV-LTR (from Rous sarcoma virus) (Gorman, C.M., *Proc. Natl. Acad. Sci. USA 79*:6777 (1982). Also useful are the promoter of the mouse metallothionein I gene (Hamer, D., *et al.*, *J. Mol. Appl. Gen. 1*:273-288 (1982)); the TK promoter of Herpes virus (McKnight, S., *Cell 31*:355-365 (1982)); the SV40 early promoter (Benoist, C., *et al.*, *Nature 290*:304-310 (1981)); and the yeast *gal4* gene promoter (Johnston, S.A., *et al.*, *Proc. Natl. Acad. Sci. (USA) 79*:6971-6975 (1982); Silver, P.A., *et al.*, *Proc. Natl. Acad. Sci. (USA) 81*:5951-5955 (1984)). Other illustrative descriptions of transcriptional factor association with promoter regions and the separate activation and DNA binding of transcription factors include: Keegan *et al.*, *Nature* (1986) *231*:699; Fields *et al.*, *Nature* (1989) 340:245; Jones, *Cell* (1990) *61*:9; Lewin, Cell (1990) *61*:1161; Ptashne *et al.*, *Nature* (1990) *346*:329; Adams *et al.*, *Cell* (1993) 72:306. The relevant disclosure of all of these above-listed references is hereby incorporated by reference.

The promoter region may further include an octamer region which may also function as a tissue specific enhancer, by interacting with certain proteins found in the specific tissue. The enhancer domain of the DNA construct of the present invention is one which is specific for the target cells to be transfected, or is highly activated by cellular factors of such target cells. Examples of vectors (plasmid or retrovirus) are disclosed in (Roy-Burman *et al.*, U.S. Patent No.

5,112,767). For a general discussion of enhancers and their actions in transcription, see, Lewin, B.M., *Genes IV*, Oxford University Press, Oxford, (1990), pp. 552-576. Particularly useful are retroviral enhancers (*e.g.*, viral LTR). The enhancer is preferably placed upstream from the promoter with which it interacts to stimulate gene expression. For use with retroviral vectors, the endogenous viral LTR may be rendered enhancer-less and substituted with other desired enhancer sequences which confer tissue specificity or other desirable properties such as transcriptional efficiency.

The nucleic acid sequences of the invention can also be chemically synthesized using standard techniques. Various methods of chemically synthesizing polydeoxynucleotides are known, including solid-phase synthesis which, like peptide synthesis, has been fully automated with commercially available DNA synthesizers (See, *e.g.*, Itakura *et al.* U.S. Pat. No. 4,598,049; Caruthers *et al.* U.S. Pat. No. 4,458,066; and Itakura U.S. Pat. Nos. 4,401,796 and 4,373,071, incorporated by reference herein).

PROTEINS AND POLYPEPTIDES

While the present disclosure exemplifies the use of a particular class of IPP, an intercellular spreading protein, particularly, the full length VP22 protein of HSV-1, it is to be understood that homologues a useful IPP, such as VP22 from other viruses or from non-viral origin, and mutants thereof that possess the characteristics disclosed herein are intended within the scope of this invention.

Thus, the present invention includes a molecular vaccine encoding a "functional derivative" of an IPP such as the intercellular spreading protein VP22. Described in terms of VP22 merely for the sake of simplicity and clarity, but not limitation, such a functional derivative is is an amino acid substitution variant, a "fragment," or a "chemical derivative" of VP22, which terms are defined below. A functional derivative retains measurable VP22-like activity, preferably that of promoting intercellular spreading and immunogenicity of one or more antigenic epitopes fused thereto. which permits its utility in accordance with the present invention. "Functional derivatives" encompass "variants" and "fragments" regardless of whether the terms are used in the conjunctive or the alternative herein.

A functional homologue must possess the above biochemical and biological activity. In view of this functional characterization, use of homologous VP22 proteins including proteins not

yet discovered, fall within the scope of the invention if these proteins have sequence similarity and the recited biochemical and biological activity.

To determine the percent identity of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In a preferred method of alignment, Cys residues are aligned.

In a preferred embodiment, the length of a sequence being compared is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, or 90% of the length of the reference sequence (*e.g.*, VP22, SEQ ID NO:5). The amino acid residues (or nucleotides) at corresponding amino acid positions (or nucleotide) positions are then compared. When a position in the first sequence is occupied by the same amino acid residue (or nucleotide) as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid "identity" is equivalent to amino acid or nucleic acid "homology"). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. In a preferred embodiment, the percent identity between two amino acid sequences is determined using the Needleman and Wunsch (*J. Mol. Biol. 48*:444-453 (1970) algorithm which has been incorporated into the GAP program in the GCG software package (available at http://www.gcg.com), using either a Blossom 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet another preferred embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package (available at http://www.gcg.com), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. In another embodiment, the percent identity between two amino acid or nucleotide sequences is determined using the algorithm of E. Meyers and W. Miller (CABIOS, 4:11-17 (1989)) which has been incorporated

into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

The nucleic acid and protein sequences of the present invention can further be used as a "query sequence" to perform a search against public databases, for example, to identify other family members or related sequences. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul *et al.* (1990) *J. Mol. Biol. 215*:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to HVP22 nucleic acid molecules. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to HVP22 protein molecules. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res. 25*:3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (*e.g.*,, XBLAST and NBLAST) can be used. See http://www.ncbi.nlm.nih.gov.

Thus, a homologue of HVP22 described above is characterized as having (a) functional activity of native HVP22 and (b) sequence similarity to a native VP22 protein (such as SEQ ID NO:5) when determined above, of at least about 20% (at the amino acid level), preferably at least about 40%, more preferably at least about 70%, even more preferably at least about 90%.

It is within the skill in the art to obtain and express such a protein using DNA probes based on the disclosed sequences of VP22. Then, the fusion protein's biochemical and biological activity can be tested readily using art-recognized methods such as those described herein, for example, a T cell proliferation, cytokine secretion or a cytolytic assay, or an *in vivo* assay of tumor protection or therapy. A biological assay of the stimulation of antigen-specific T cell reactivity will indicate whether the homologue has the requisite activity to qualify as a "functional" homologue.

A "variant" of a HVP22 refers to a molecule substantially identical to either the full protein or to a fragment thereof in which one or more amino acid residues have been replaced (substitution variant) or which has one or several residues deleted (deletion variant) or added (addition variant). A "fragment" of HVP22 refers to any subset of the molecule, that is, a shorter polypeptide of the full-length protein.

A number of processes can be used to generate fragments, mutants and variants of the isolated DNA sequence. Small subregions or fragments of the nucleic acid encoding the spreading protein, for example 1-30 bases in length, can be prepared by standard, chemical synthesis. Antisense oligonucleotides and primers for use in the generation of larger synthetic fragment.

A preferred group of variants are those in which at least one amino acid residue and preferably, only one, has been substituted by different residue. For a detailed description of protein chemistry and structure, see Schulz, GE *et al.*, *Principles of Protein Structure*, Springer-Verlag, New York, 1978, and Creighton, T.E., *Proteins: Structure and Molecular Properties*, W.H. Freeman & Co., San Francisco, 1983, which are hereby incorporated by reference. The types of substitutions that may be made in the protein molecule may be based on analysis of the frequencies of amino acid changes between a homologous protein of different species, such as those presented in Table 1-2 of Schulz *et al. (supra)* and Figure 3-9 of Creighton (*supra*). Based on such an analysis, conservative substitutions are defined herein as exchanges within one of the following five groups:

1	Small aliphatic, nonpolar or slightly polar residues	Ala, Ser, Thr (Pro, Gly);
2	Polar, negatively charged residues and their amides	Asp, Asn, Glu, Gln;
3	Polar, positively charged residues	His, Arg, Lys;
4	Large aliphatic, nonpolar residues	Met, Leu, Ile, Val (Cys)
5	Large aromatic residues	Phe, Tyr, Trp.

The three amino acid residues in parentheses above have special roles in protein architecture. Gly is the only residue lacking a side chain and thus imparts flexibility to the chain. Pro, because of its unusual geometry, tightly constrains the chain. Cys can participate in disulfide bond formation, which is important in protein folding.

More substantial changes in biochemical, functional (or immunological) properties are made by selecting substitutions that are less conservative, such as between, rather than within, the above five groups. Such changes will differ more significantly in their effect on maintaining (a) the structure of the peptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Examples of such substitutions are (i) substitution of Gly and/or

Pro by another amino acid or deletion or insertion of Gly or Pro; (ii) substitution of a hydrophilic residue, *e.g.*, Ser or Thr, for (or by) a hydrophobic residue, *e.g.*,, Leu, Ile, Phe, Val or Ala; (iii) substitution of a Cys residue for (or by) any other residue; (iv) substitution of a residue having an electropositive side chain, *e.g.*,, Lys, Arg or His, for (or by) a residue having an electronegative charge, *e.g.*,, Glu or Asp; or (v) substitution of a residue having a bulky side chain, *e.g.*, Phe, for (or by) a residue not having such a side chain, *e.g.*, Gly.

Most acceptable deletions, insertions and substitutions according to the present invention are those that do not produce radical changes in the characteristics of the IPP, *e.g.*, HVP22, in terms of its intercellular spreading activity and its ability to stimulate antigen specific T cell reactivity to an antigenic epitope or epitopes that are fused to the spreading protein. However, when it is difficult to predict the exact effect of the substitution, deletion or insertion in advance of doing so, one skilled in the art will appreciate that the effect can be evaluated by routine screening assays such as those described here, without requiring undue experimentation.

Whereas shorter chain variants can be made by chemical synthesis, for the present invention, the preferred longer chain variants are typically made by site-specific mutagenesis of the nucleic acid encoding the IPP, expression of the variant nucleic acid in cell culture, and, optionally, purification of the polypeptide from the cell culture, for example, by immunoaffinity chromatography using specific antibody immobilized to a column (to absorb the variant by binding to at least one epitope).

Chemical Derivatives

"Chemical derivatives" of the IPP, *e.g.*, HVP22, or a fusion polypeptide thereof, contain additional chemical moieties not normally a part of the protein. Covalent modifications of the polypeptide are included within the scope of this invention. Such derivatized moieties may improve the solubility, absorption, biological half life, and the like. Moieties capable of mediating such effects are disclosed, for example, in *Remington's Pharmaceutical Sciences*, 16th ed., Mack Publishing Co., Easton, PA (1980).

Such modifications may be introduced into the molecule by reacting targeted amino acid residues of the polypeptide with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues. Another modification is cyclization of the protein. Examples of chemical derivatives of the polypeptide follow.

Lysinyl and amino terminal residues are derivatized with succinic or other carboxylic acid anhydrides. Derivatization with a cyclic carboxylic anhydride has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing amino-containing residues include imidoesters such as methyl picolinimidate; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4 pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Carboxyl side groups, aspartyl or glutamyl, may be selectively modified by reaction with carbodiimides (R-N=C=N-R') such as 1-cyclohexyl-3-(2-morpholinyl-(4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues can be converted to asparaginyl and glutaminyl residues by reaction with ammonia.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the amino group of lysine (Creighton, *supra,* pp. 79-86), acetylation of the N-terminal amine, and amidation of the C-terminal carboxyl groups.

Also included are peptides wherein one or more D-amino acids are substituted for one or more L-amino acids.

Multimeric Peptides

The present invention includes longer polypeptides in which a basic peptidic sequence obtained from the sequence of either the IPP, such as HVP22, or the antigenic polypeptide or peptide unit, is repeated from about two to about 100 times, with or without intervening spacers or linkers. It is understood that such multimers may be built from any of the peptide variants defined herein. Moreover, a peptide multimer may comprise different combinations of peptide monomers and the disclosed substitution variants thereof. Such oligomeric or multimeric peptides can be made by chemical synthesis or by recombinant DNA techniques as discussed herein. When produced chemically, the oligomers preferably have from 2-8 repeats of the basic peptide sequence. When produced recombinantly, the multimers may have as many repeats as the expression system permits, for example from two to about 100 repeats.

In tandem multimers, preferably dimers and trimers, of the fusion polypeptide, the chains bonded by interchain disulfide bonds or other "artificial" covalent bonds between the chains such that the chains are "side-by-side" rather than "end to end."

THERAPEUTIC COMPOSITIONS AND THEIR ADMINISTRATION

A vaccine composition comprising the nucleic acid encoding the fusion polypeptide, or a cell expressing this nucleic acid is administered to a mammalian subject, preferably a human. The vaccine composition is administered in a pharmaceutically acceptable carrier in a biologically effective or a therapeutically effective amount . The composition may be given alone or in combination with another protein or peptide such as an immunostimulatory molecule. Treatment may include administration of an adjuvant, used in its broadest sense to include any nonspecific immune stimulating compound such as an interferon. Adjuvants contemplated herein include resorcinols, non-ionic surfactants such as polyoxyethylene oleyl ether and n-hexadecyl polyethylene ether.

A therapeutically effective amount is a dosage that, when given for an effective period of time, achieves the desired immunological or clinical effect.

A therapeutically active amount of a nucleic acid encoding the fusion polypeptide may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the peptide to elicit a desired response in the individual. Dosage regimes may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation. A therapeutically effective amounts of the protein, in cell associated form may be stated in terms of the protein or cell equivalents.

Thus an effective amount is between about 1 nanogram and about 10 milligram per kilogram of body weight of the recipient, more preferably between about 0.1 μg and 1 μg/kg. Dosage forms suitable for internal administration preferably contain (for the latter dose range) from about 0.01 μg to 100 μg of active ingredient (nucleic acid or polypeptide) per unit. The active ingredient may vary from 0.5 to 95% by weight based on the total weight of the composition. Alternatively, an effective dose of cells expressing the nucleic acid is between about 10^4 and 10^8 cells. Those skilled in the art of immunotherapy will be able to adjust these doses without undue experimentation.

The active compound may be administered in a convenient manner, *e.g.*, injection or infusion by a convenient and effective route. Preferred routes include subcutaneous, intradermal, intravenous and intramuscular routes. Other possible routes include oral administration, intrathecal, inhalation, transdermal application, or rectal administration. For the treatment of tumors which have not been completely resected, direct intratumoral or peritumoral injection is also intended.

Depending on the route of administration, the active compound may be coated in a material to protect the compound from the action of enzymes, acids and other natural conditions which may inactivate the compound. Thus it may be necessary to coat the composition with, or co-administer the composition with, a material to prevent its inactivation. For example, an enzyme inhibitors of nucleases or proteases (*e.g.*, pancreatic trypsin inhibitor, diisopropylfluorophosphate and trasylol).or in an appropriate carrier such as liposomes (including water-in-oil-in-water emulsions as well as conventional liposomes (Strejan *et al.*, (1984) *J. Neuroimmunol* 7:27).

As used herein "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the therapeutic compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

Preferred pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Pharmaceutical compositions suitable for injection include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. Isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride may be included in the pharmaceutical composition. In all cases, the composition should be sterile and should be fluid. It should be stable under the conditions of manufacture and storage and must include preservatives that prevent contamination with microorganisms such as bacteria and fungi. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms.

The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants.

Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like.

Parenteral compositions are preferably formulated in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form refers to physically discrete units suited as unitary dosages for a mammalian subject; each unit contains a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

For lung instillation, aerosolized solutions are used. In a sprayable aerosol preparations, the active protein may be in combination with a solid or liquid inert carrier material. This may also be packaged in a squeeze bottle or in admixture with a pressurized volatile, normally gaseous propellant. The aerosol preparations can contain solvents, buffers, surfactants, and antioxidants in addition to the protein of the invention.

Other pharmaceutically acceptable carriers for the nucleic acid vaccine compositions according to the present invention are liposomes, pharmaceutical compositions in which the active protein is contained either dispersed or variously present in corpuscles consisting of aqueous concentric layers adherent to lipidic layers. The active protein is preferably present in the aqueous layer and in the lipidic layer, inside or outside, or, in any event, in the non-homogeneous system generally known as a liposomic suspension. The hydrophobic layer, or lipidic layer, generally, but not exclusively, comprises phospholipids such as lecithin and sphingomyelin, steroids such as cholesterol, more or less ionic surface active substances such as

dicetylphosphate, stearylamine or phosphatidic acid, and/or other materials of a hydrophobic nature.

ANTIGENS ASSOCIATED WITH PATHOGENS

A major utility for the present invention is the use of the present nucleic acid compositions in therapeutic vaccine for cancer and for major chronic viral infections that cause morbidity and mortality worldwide. Such vaccines are designed to eliminate infected cells - this requires T cell responses as antibodies are often ineffective. The vaccines of the present invention, include, the antigenic epitope itself and an IPP such as an intercellular spreading protein like HVP22. In addition to the specific antigens and first IPP polypeptide in the present vectors as employed in the Examples, the present invention is intended to encompass

(a) use of additional vectors such as naked DNA, naked RNA, self replicating RNA replicons and viruses including vaccinia, adenoviruses, adeno-associated virus (AAV), lentiviruses and RNA alphaviruses;

(b) an additional IPP such as HSP70, calreticulin, the extracellular domain of FL, , domain II of Pseudomonas exotoxin ETA; and/or

(c) a costimulatory signal, such as a B7 family protein, including B7-DC (see commonly assigned U.S. patent application Serial No. 09/794,210 which is incorporated by reference in its entirety), B7.1, B7.2, soluble CD40, *etc.*).

Preferred antigens are preferably epitopes of pathogenic microorganisms against which the host is defended by effector T cells responses, including cytotoxic T lymphocyte (CTL) and delayed type hypersensitivity. These typically include viruses, intracellular parasites such as malaria, and bacteria that grow intracellularly such as mycobacteria and listeria. Thus, the types of antigens included in the vaccine compositions of this invention are any of those associated with such pathogens (in addition, of course, to tumor-specific antigens). It is noteworthy that some viral antigens are also tumor antigens in the case where the virus is a causative factor in cancer.

In fact, the two most common cancers worldwide, hepatoma and cervical cancer, are associated with viral infection. Hepatitis B virus(HBV) (Beasley, R.P. *et al.*, *Lancet* **2**, 1129-1133 (1981) has been implicated as etiologic agent of hepatomas. 80-90% of cervical cancers express the E6 and E7 antigens (exemplified herein) from one of four "high risk" human

papillomavirus types: HPV-16, HPV-18, HPV-31 and HPV-45 (Gissmann, L. *et al.*, *Ciba Found Symp.* **120**, 190-207 (1986); Beaudenon, S., *et al. Nature* **321**, 246-249 (1986). The HPV E6 and E7 antigens are the most promising targets for virus associated cancers in immunocompetent individuals because of their ubiquitous expression in cervical cancer. In addition to their importance as targets for therapeutic cancer vaccines, virus associated tumor antigens are also ideal candidates for prophylactic vaccines. Indeed, introduction of prophylactic HBV vaccines in Asia have decreased the incidence of hepatoma (Chang, M.H., *et al. New Engl. J. Med.* **336**, 1855-1859 (1997), representing a great impact on cancer prevention.

Among the most important viruses in chronic human viral infections are human papillomavirus (HPV) hepatitis B virus (HBV), hepatitis C Virus (HCV), human immunodeficiency virus (HIV), Epstein Barr Virus (EBV) and herpes simplex virus (HSV).

In addition to its applicability to human cancer and infectious diseases,, the present invention is also intended for use in treating animal diseases in the veterinary medicine context. Thus, the approaches described herein may be readily applied by one skilled in the art to treatment of veterinary herpesvirus infections including equine herpesviruses, bovine herpesviruses, Marek's disease virus in chickens and other fowl; animal retroviral diseases; pseudorabies and rabies and the like.

The following references set forth principles and current information in the field of basic, medical and veterinary virology and are incorporated by reference: *Fields Virology*, Fields, BN *et al.*, eds., Lippincott Williams & Wilkins, NY, 1996; *Principles of Virology: Molecular Biology, Pathogenesis, and Control, Flint*, S.J. *et al.*, eds., Amer Society for Microbiology, Washington, 1999; *Principles and Practice of Clinical Virology*, 4th Edition, Zuckerman. A.J. *et al.*, eds, John Wiley & Sons, NY, 1999; *The Hepatitis C Viruses*, by Hagedorn, CH *et al.*, eds., Springer Verlag, 1999; *Hepatitis B Virus: Molecular Mechanisms in Disease and Novel Strategies for Therapy,* Koshy, R. *et al.*, eds,, World Scientific Pub Co, 1998; *Veterinary Virology*, Murphy, F.A. *et al.*, eds., Academic Press, NY, 1999; *Avian Viruses: Function and Control*, Ritchie, B.W., Iowa State University Press, Ames , 2000; *Virus Taxonomy: Classification and Nomenclature of Viruses: Seventh Report of the International Committee on Taxonomy of Viruses,* by M. H. V. Van Regenmortel, MHV *et al.*, eds., Academic Press; NY, 2000.

DELIVERY OF VACCINE NUCLEIC ACID TO CELLS AND ANIMALS

The Examples below describe certain preferred approaches to delivery of the vaccines of the present invention.

DNA delivery, for example to effect what is generally known as "gene therapy" involves introduction of a "foreign" DNA into a cell and ultimately, into a live animal. Several general strategies for gene therapy have been studied and have been reviewed extensively (Yang, N-S., *Crit. Rev. Biotechnol. 12*:335-356 (1992); Anderson, W.F., *Science 256*:808-813 (1992); Miller, A.S., *Nature 357*:455-460 (1992); Crystal, R.G., *Amer. J. Med. 92(suppl 6A)*:44S-52S (1992); Zwiebel, J.A. *et al.*, *Ann. N.Y. Acad. Sci. 618*:394-404 (1991); McLachlin, J.R. *et al.*, *Prog. Nucl. Acid Res. Molec. Biol. 38*:91-135 (1990); Kohn, D.B. *et al.*, *Cancer Invest. 7*:179-192 (1989), which references are herein incorporated by reference in their entirety).

One approach comprises nucleic acid transfer into primary cells in culture followed by autologous transplantation of the *ex vivo* transformed cells into the host, either systemically or into a particular organ or tissue.

For accomplishing the objectives of the present invention, nucleic acid therapy would be accomplished by direct transfer of a the functionally active vectors into mammalian somatic tissue or organ *in vivo*. Nucleic acid or replicon transfer can be achieved using a number of approaches described below. These systems can be tested for successful expression *in vitro* by use of a selectable marker (*e.g.*, G418 resistance) to select transfected clones expressing the DNA, followed by detection of the presence of the antigen-containing expression product (after treatment with the inducer in the case of an inducible system) using an antibody to the product in an appropriate immunoassay. Efficiency of the procedure, including DNA uptake, integration and stability of integrated DNA, can be improved using known methods, and co-transfection using high molecular weight mammalian DNA as a "carrier".

Examples of successful "gene transfer" reported in the art include: (a) direct injection of plasmid DNA into mouse muscle tissues, which led to expression of marker genes for an indefinite period of time (Wolff, J.A. *et al.*, *Science 247*:1465 (1990); Acsadi, G. *et al.*, *The New Biologist 3*:71 (1991)); (b) retroviral vectors are effective for *in vivo* and *in situ* infection of blood vessel tissues; (c) portal vein injection and direct injection of retrovirus preparations into liver effected gene transfer and expression *in vivo* (Horzaglou, M. *et al.*, *J. Biol. Chem. 265*:17285 (1990); Koleko, M. *et al.*, *Human Gene Therapy 2*:27 (1991); Ferry, N. *et al.*, *Proc.*

Natl. Acad. Sci. USA 88:8387 (1991)); (d) intratracheal infusion of recombinant adenovirus into lung tissues was effective for *in vivo* transfer and prolonged expression of foreign genes in lung respiratory epithelium (Rosenfeld, M.A. *et al.*, *Science 252*:431 (1991); (e) Herpes simplex virus vectors achieved *in vivo* gene transfer into brain tissue (Ahmad, F. *et al.*, eds, *Miami Short Reports - Advances in Gene Technology: The Molecular Biology of Human Genetic Disease*, Vol 1, Boehringer Manneheim1 Biochemicals, USA, 1991).

Retroviral-mediated human therapy utilizes amphotrophic, replication-deficient retrovirus systems (Temin, H.M., *Human Gene Therapy 1*:111 (1990); Temin *et al.*, U.S. Patent 4,980,289; Temin *et al.*, U.S. Patent 4,650,764; Temin *et al.*, U.S. Patent No. 5,124,263; Wills, J.W. U.S. Patent 5,175,099; Miller, A.D., U.S. Patent No. 4,861,719). Such vectors have been used to introduce functional DNA into human cells or tissues, for example, the adenosine deaminase gene into lymphocytes, the NPT-II gene and the gene for tumor necrosis factor into tumor infiltrating lymphocytes. Retrovirus-mediated gene delivery generally requires target cell proliferation for gene transfer (Miller, D.G. *et al.*, *Mol. Cell. Biol. 10*:4239 (1990). This condition is met by certain of the preferred target cells into which the present DNA molecules are to be introduced, *i.e.*, actively growing tumor cells. Gene therapy of cystic fibrosis using transfection by plasmids using any of a number of methods and by retroviral vectors has been described by Collins *et al.*, U.S. Patent 5,240,846.

Other vectors that may be used in conjunction with the present vectors to include DNA packaged into retrovirus vectors using packaging cell lines that produce replication-defective retroviruses, as is well-known in the art (see, for example, Cone, R.D. *et al.*, *Proc. Natl. Acad. Sci. USA* 81:6349-6353 (1984); Mann, R.F. *et al.*, *Cell* 33:153-159 (1983); Miller, A.D. *et al.*, *Molec. Cell. Biol.* 5:431-437 (1985),; Sorge, J., *et al.*, *Molec. Cell. Biol.* 4:1730-1737 (1984); Hock, R.A. *et al.*, *Nature* 320:257 (1986); Miller, A.D. *et al.*, *Molec. Cell. Biol.* 6:2895-2902 (1986). Newer packaging cell lines which are efficient an safe for gene transfer have also been described (Bank *et al.*, U.S. 5,278,056.

This approach can be utilized in a site specific manner to deliver the retroviral vector to the tissue or organ of choice. Thus, for example, a catheter delivery system can be used (Nabel, EG *et al.*, *Science 244*:1342 (1989)). Such methods, using either a retroviral vector or a liposome vector, are particularly useful to deliver the nucleic acid to be expressed to a blood vessel wall, or into the blood circulation of a tumor.

Other virus vectors may also be used, including recombinant adenoviruses (Horowitz, M.S., In: *Virology*, Fields, BN *et al.*, eds, Raven Press, New York, 1990, p. 1679; Berkner, K.L., *Biotechniques 6*:616 9191988), Strauss, S.E., In: *The Adenoviruses*, Ginsberg, HS, ed., Plenum Press, New York, 1984, chapter 11), herpes simplex virus (HSV) for neuron-specific delivery and persistence. Advantages of adenovirus vectors for human gene therapy include the fact that recombination is rare, no human malignancies are known to be associated with such viruses, the adenovirus genome is double stranded DNA which can be manipulated to accept foreign genes of up to 7.5 kb in size, and live adenovirus is a safe human vaccine organisms. Adeno-associated virus is also useful for human therapy (Samulski, R.J. *et al.*, *EMBO J. 10*:3941 (1991) according to the present invention.

Vaccinia virus, which can be rendered non-replicating (U.S. Patents 5,225,336; 5,204,243; 5,155,020; 4,769,330; Sutter, G *et al.*, *Proc. Natl. Acad. Sci. USA* (1992) *89*:10847-10851; Fuerst, T.R. *et al.*, *Proc. Natl. Acad. Sci. USA* (1989) *86*:2549-2553; Falkner F.G. *et al.*; *Nucl. Acids Res* (1987) *15*:7192; Chakrabarti, S *et al.*, *Molec. Cell. Biol.* (1985) *5*:3403-3409). Descriptions of recombinant vaccinia viruses and other viruses containing heterologous DNA and their uses in immunization and DNA therapy are reviewed in: Moss, B., *Curr. Opin. Genet. Dev.* (1993) *3*:86-90; Moss, B. *Biotechnology* (1992) *20*: 345-362; Moss, B., *Curr Top Microbiol Immunol* (1992) *158*:25-38; Moss, B., *Science* (1991) *252*:1662-1667; Piccini, A *et al.*, *Adv. Virus Res.* (1988) 34:43-64; Moss, B. *et al.*, *Gene Amplif Anal* (1983) 3:201-213.

In addition engineered bacteria may be used as additional vectors. A number of bacterial strains including *Salmonella*, BCG and *Listeria monocytogenes*(LM) (Hoiseth & Stocker, *Nature* **291**, 238-239 (1981); Poirier, TP *et al. J. Exp. Med.* **168**, 25-32 (1988); (Sadoff, J.C., *et al., Science* **240**, 336-338 (1988); Stover, C.K., *et al., Nature* **351**, 456-460 (1991); Aldovini, A. *et al.,, Nature* **351**, 479-482 (1991); Schafer, R., *et al., J. Immunol.* **149**, 53-59 (1992); Ikonomidis, G. *et al., J. Exp. Med.* **180**, 2209-2218 (1994)). These organisms display two promising characteristics for use as vaccine vectors: (1) enteric routes of infection, providing the possibility of oral vaccine delivery; and (2) infection of monocytes/macrophages thereby targeting antigens to professional APCs.

In addition to the forms of nucleic acid transfer *in vivo* described above, physical means well-known in the art can be used for direct transfer of DNA, including administration of

plasmid DNA (Wolff *et al.*, 1990, *supra*) and particle-bombardment mediated gene transfer (Yang, N.-S., *et al.*, *Proc. Natl. Acad. Sci. USA 87*:9568 (1990); Williams, R.S. *et al.*, *Proc. Natl. Acad. Sci. USA 88*:2726 (1991); Zelenin, A.V. *et al.*, *FEBS Lett. 280*:94 (1991); Zelenin, A.V. *et al.*, *FEBS Lett. 244*:65 (1989); Johnston, S.A. *et al.*, *In Vitro Cell. Dev. Biol. 27*:11 (1991)). Furthermore, electroporation, a well-known means to transfer genes into cell *in vitro*, can be used to transfer DNA molecules according to the present invention to tissues *in vivo* (Titomirov, A.V. *et al.*, *Biochim. Biophys. Acta 1088*:131 ((1991)).

"Carrier mediated gene transfer" has also been described (Wu, C.H. *et al.*, *J. Biol. Chem. 264*:16985 (1989); Wu, G.Y. *et al.*, *J. Biol. Chem. 263*:14621 (1988); Soriano, P. *et al.*, *Proc. Natl. Acad. Sci. USA 80*:7128 (1983); Wang, C-Y. *et al.*, *Proc. Natl. Acad. Sci. USA 84*:7851 (1982); Wilson, J.M. *et al.*, *J. Biol. Chem. 267*:963 (1992)). Preferred carriers are targeted liposomes (Nicolau, C. *et al.*, *Proc. Natl. Acad. Sci. USA 80*:1068 (1983); Soriano *et al.*, *supra*) such as immunoliposomes, which can incorporate acylated mAbs into the lipid bilayer (Wang *et al.*, *supra*). Polycations such as asialoglycoprotein/polylysine may be used, where the conjugate includes a molecule which recognizes the target tissue (e.g., asialoorosomucoid for liver) and a DNA binding compound to bind to the DNA to be transfected. Polylysine is an example of a DNA binding molecule which binds DNA without damaging it. This conjugate is then complexed with plasmid DNA according to the present invention for transfer.

DNA used for transfection or microinjection may be prepared using methods well-known in the art, for example using the Quiagen procedure (Quiagen), followed by DNA purification using known methods, such as the methods exemplified herein.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.

EXAMPLE I

Materials and Methods

Cell Lines

The packaging cell line (987dlsplit #24) has been described previously (Polo *et al.*, *supra*) and was grown in DMEM supplemented with 10% FBS, antibiotics and G418. The production and maintenance of TC-1 cells has been described previously (Lin, KY *et al.*, 1996, *Cancer Research. 56*:21-26). On the day of tumor challenge, TC-1 cells were harvested by trypsinization,

washed twice with 1X Hanks buffered salt solution (HBSS), and finally resuspended in 1X HBSS to the designated concentration for injection. Baby hamster kidney (BHK21) cells were obtained from the ATCC (Rockville, MD) and grown in Glasgow MEM supplemented with 5% FBS, 10% tryptose phosphate broth, 2 mM glutamine, and antibiotics.

Plasmid DNA Constructs

The Sindbis virus RNA replicon vector, SINrep5, previously described (Bredenbeek, PJ *et al.*, *supra*) was provided by Dr. Charles Rice at the Washington University School of Medicine, St. Louis.

The sequence of SINrep5 self replicating replicon (SEQ ID NO:23) is shown below and includes cloning sites

ATTGACGGCG TAGTACACAC TATTGAATCA AACAGCCGAC CAATTGCACT ACCATCACAA TGGAGAAGCC
AGTAGTAAAC GTAGACGTAG ACCCCCAGAG TCCGTTTGTC GTGCAACTGC AAAAAAGCTT CCCGCAATTT
GAGGTAGTAG CACAGCAGGT CACTCCAAAT GACCATGCTA ATGCCAGAGC ATTTTCGCAT CTGGCCAGTA
AACTAATCGA GCTGGAGGTT CCTACCACAG CGACGATCTT GGACATAGGC AGCGCACCGG CTCGTAGAAT
GTTTTCCGAG CACCAGTATC ATTGTGTCTG CCCCATGCGT AGTCCAGAAG ACCCGGACCG CATGATGAAA
TACGCCAGTA AACTGGCGGA AAAAGCGTGC AAGATTACAA ACAAGAACTT GCATGAGAAG ATTAAGGATC
TCCGGACCGT ACTTGATACG CCGGATGCTG AAACACCATC GCTCTGCTTT CACAACGATG TTACCTGCAA
CATGCGTGCC GAATATTCCG TCATGCAGGA CGTGTATATC AACGCTCCCG GAACTATCTA TCATCAGGCT
ATGAAAGGCG TGCGGACCCT GTACTGGATT GGCTTCGACA CCACCCAGTT CATGTTCTCG GCTATGGCAG
GTTCGTACCC TGCGTACAAC ACCAACTGGG CCGACGAGAA AGTCCTTGAA GCGCGTAACA TCGGACTTTG
CAGCACAAAG CTGAGTGAAG GTAGGACAGG AAAATTGTCG ATAATGAGGA AGAAGGAGTT GAAGCCCGGG
TCGCGGGTTT ATTTCTCCGT AGGATCGACA CTTTATCCAG AACACAGAGC CAGCTTGCAG AGCTGGCATC
TTCCATCGGT GTTCCACTTG AATGGAAAGC AGTCGTACAC TTGCCGCTGT GATACAGTGG TGAGTTGCGA
AGGCTACGTA GTGAAGAAAA TCACCATCAG TCCCGGGATC ACGGGAGAAA CCGTGGGATA CGCGGTTACA
CACAATAGCG AGGGCTTCTT GCTATGCAAA GTTACTGACA CAGTAAAAGG AGAACGGGTA TCGTTCCCTG
TGTGCACGTA CATCCCGGCC ACCATATGCG ATCAGATGAC TGGTATAATG GCCACGGATA TATCACCTGA
CGATGCACAA AAACTTCTGG TTGGGCTCAA CCAGCGAATT GTCATTAACG GTAGGACTAA CAGGAACACC
AACACCATGC AAAATTACCT TCTGCCGATC ATAGCACAAG GGTTCAGCAA ATGGGCTAAG GAGCGCAAGG
ATGATCTTGA TAACGAGAAA ATGCTGGGTA CTAGAGAACG CAAGCTTACG TATGGCTGCT TGTGGGCGTT
TCGCACTAAG AAAGTACATT CGTTTTATCG CCCACCTGGA ACGCAGACCT GCGTAAAAGT CCCAGCCTCT
TTTAGCGCTT TTCCCATGTC GTCCGTATGG ACGACCTCTT TGCCCATGTC GCTGAGGCAG AAATTGAAAC
TGGCATTGCA ACCAAAGAAG GAGGAAAAAC TGCTGCAGGT CTCGGAGGAA TTAGTCATGG AGGCCAAGGC
TGCTTTTGAG GATGCTCAGG AGGAAGCCAG AGCGGAGAAG CTCCGAGAAG CACTTCCACC ATTAGTGGCA
GACAAAGGCA TCGAGGCAGC CGCAGAAGTT GTCTGCGAAG TGGAGGGGCT CCAGGCGGAC ATCGGAGCAG
CATTAGTTGA AACCCCGCGC GGTCACGTAA GGATAATACC TCAAGCAAAT GACCGTATGA TCGGACAGTA
TATCGTTGTC TCGCCAAACT CTGTGCTGAA GAATGCCAAA CTCGCACCAG CGCACCCGCT AGCAGATCAG
GTTAAGATCA TAACACACTC CGGAAGATCA GGAAGGTACG CGGTCGAACC ATACGACGCT AAAGTACTGA
TGCCAGCAGG AGGTGCCGTA CCATGGCCAG AATTCCTAGC ACTGAGTGAG AGCGCCACGT TAGTGTACAA
CGAAAGAGAG TTTGTGAACC GCAAACTATA CCACATTGCC ATGCATGGCC CCGCCAAGAA TACAGAAGAG
GAGCAGTACA AGGTTACAAA GGCAGAGCTT GCAGAAACAG AGTACGTGTT TGACGTGGAC AAGAAGCGTT
GCGTTAAGAA GGAAGAAGCC TCAGGTCTGG TCCTCTCGGG AGAACTGACC AACCCTCCCT ATCATGAGCT
AGCTCTGGAG GGACTGAAGA CCCGACCTGC GGTCCCGTAC AAGGTCGAAA CAATAGGAGT GATAGGCACA
CCGGGGTCGG GCAAGTCAGC TATTATCAAG TCAACTGTCA CGGCACGAGA TCTTGTTACC AGCGGAAAGA
AAGAAAATTG TCGCGAAATT GAGGCCGACG TGCTAAGACT GAGGGGTATG CAGATTACGT CGAAGACAGT
AGATTCGGTT ATGCTCAACG GATGCCACAA AGCCGTAGAA GTGCTGTACG TTGACGAAGC GTTCGCGTGC
CACGCAGGAG CACTACTTGC CTTGATTGCT ATCGTCAGGC CCCGCAAGAA GGTAGTACTA TGCGGAGACC
CCATGCAATG CGGATTCTTC AACATGATGC AACTAAAGGT ACATTTCAAT CACCCTGAAA AAGACATATG
CACCAAGACA TTCTACAAGT ATATCTCCCG GCGTTGCACA CAGCCAGTTA CAGCTATTGT ATCGACACTG
CATTACGATG GAAAGATGAA AACCACGAAC CCGTGCAAGA AGAACATTGA AATCGATATT ACAGGGGCCA
CAAAGCCGAA GCCAGGGGAT ATCATCCTGA CATGTTTCCG CGGGTGGGTT AAGCAATTGC AAATCGACTA
TCCCGGACAT GAAGTAATGA CAGCCGCGGC CTCACAAGGG CTAACCAGAA AAGGAGTGTA TGCCGTCCGG
CAAAAAGTCA ATGAAAACCC ACTGTACGCG ATCACATCAG AGCATGTGAA CGTGTTGCTC ACCCGCACTG
AGGACAGGCT AGTGTGGAAA ACCTTGCAGG GCGACCCATG GATTAAGCAG CCCACTAACA TACCTAAAGG
AAACTTTCAG GCTACTATAG AGGACTGGGA AGCTGAACAC AAGGGAATAA TTGCTGCAAT AAACAGCCCC
ACTCCCCGTG CCAATCCGTT CAGCTGCAAG ACCAACGTTT GCTGGGCGAA AGCATTGGAA CCGATACTAG
CCACGGCCGG TATCGTACTT ACCGGTTGCC AGTGGAGCGA ACTGTTCCCA CAGTTTGCGG ATGACAAACC
ACATTCGGCC ATTTACGCCT TAGACGTAAT TTGCATTAAG TTTTTCGGCA TGGACTTGAC AAGCGGACTG
TTTTCTAAAC AGAGCATCCC ACTAACGTAC CATCCCGCCG ATTCAGCGAG GCCGGTAGCT CATTGGGACA
ACAGCCCAGG AACCCGCAAG TATGGGTACG ATCACGCCAT TGCCGCCGAA CTCTCCCGTA GATTTCCGGT

GTTCCAGCTA GCTGGGAAGG GCACACAACT TGATTTGCAG ACGGGGAGAA CCAGAGTTAT CTCTGCACAG
CATAACCTGG TCCCGGTGAA CCGCAATCTT CCTCACGCCT TAGTCCCCGA GTACAAGGAG AAGCAACCCG
GCCCGGTCAA AAAATTCTTG AACCAGTTCA AACACCACTC AGTACTTGTG GTATCAGAGG AAAAAATTGA
AGCTCCCCGT AAGAGAATCG AATGGATCGC CCCGATTGGC ATAGCCGGTG CAGATAAGAA CTACAACCTG
GCTTTCGGGT TTCCGCCGCA GGCACGGTAC GACCTGGTGT TCATCAACAT TGGAACTAAA TACAGAAACC
ACCACTTTCA GCAGTGCGAA GACCATGCGG CGACCTTAAA AACCCTTTCG CGTTCGGCCC TGAATTGCCT
TAACCCAGGA GGCACCCTCG TGGTGAAGTC CTATGGCTAC GCCGACCGCA ACAGTGAGGA CGTAGTCACC
GCTCTTGCCA GAAAGTTTGT CAGGGTGTCT GCAGCGAGAC CAGATTGTGT CTCAAGCAAT ACAGAAATGT
ACCTGATTTT CCGACAACTA GACAACAGCC GTACACGGCA ATTCACCCCG CACCATCTGA ATTGCGTGAT
TTCGTCCGTG TATGAGGGTA CAAGAGATGG AGTTGGAGCC GCGCCGTCAT ACCGCACCAA AAGGGAGAAT
ATTGCTGACT GTCAAGAGGA AGCAGTTGTC AACGCAGCCA ATCCGCTGGG TAGACCAGGC GAAGGAGTCT
GCCGTGCCAT CTATAAACGT TGGCCGACCA GTTTTACCGA TTCAGCCACG GAGACAGGCA CCGCAAGAAT
GACTGTGTGC CTAGGAAAGA AAGTGATCCA CGCGGTCGGC CCTGATTTCC GGAAGCACCC AGAAGCAGAA
GCCTTGAAAT TGCTACAAAA CGCCTACCAT GCAGTGGCAG ACTTAGTAAA TGAACATAAC ATCAAGTCTG
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CTTGACAACC GCGCTAGACA GAACTGACGC GGACGTAACC ATCTATTGCC TGGATAAGAA GTGGAAGGAA
AGAATCGACG CGGCACTCCA ACTTAAGGAG TCTGTAACAG AGCTGAAGGA TGAAGATATG GAGATCGACG
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ATTGTATTCG TACTTCGAAG GCACCAAATT CCATCAAGCA GCAAAAGACA TGGCGGAGAT AAAGGTCCTG
TTCCCTAATG ACCAGGAAAG TAATGAACAA CTGTGTGCCT ACATATTGGG TGAGACCATG GAAGCAATCC
GCGAAAAGTG CCCGGTCGAC CATAACCCGT CGTCTAGCCC GCCCAAAACG TTGCCGTGCC TTTGCATGTA
TGCCATGACG CCAGAAAGGG TCCACAGACT TAGAAGCAAT AACGTCAAAG AAGTTACAGT ATGCTCCTCC
ACCCCCCTTC CTAAGCACAA AATTAAGAAT GTTCAGAAGG TTCAGTGCAC GAAAGTAGTC CTGTTTAATC
CGCACACTCC CGCATTCGTT CCCGCCCGTA AGTACATAGA AGTGCCAGAA CAGCCTACCG CTCCTCCTGC
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GGTGGTGGTG GCTGACGTTC ATGCCGTCCA AGAGCCTGCC CCTATTCCAC CGCCAAGGCT AAAGAAGATG
GCCCGCCTGG CAGCGGCAAG AAAAGAGCCC ACTCCACCGG CAAGCAATAG CTCTGAGTCC CTCCACCTCT
CTTTTGGTGG GGTATCCATG TCCCTCGGAT CAATTTTCGA CGGAGAGACG GCCCGCCAGG CAGCGGTACA
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TGAAACAAAG GCGTGGTTTA GAGTAGGTAT AACAGGCACT TTAGCAGTGG CCGTGACGAC CCGGTATGAG
GTAGACAATA TTACACCTGT CCTACTGGCA TTGAGAACTT TTGCCCAGAG CAAAAGAGCA TTCCAAGCCA
TCAGAGGGGA AATAAAGCAT CTCTACGGTG GTCCTAAATA GTCAGCATAG TACATTTCAT CTGACTAATA
CTACAACACC ACCACCTCTA GACGCGTAGA TCTCACGTGA GCATGCAGGC CTTGGGCCCA ATGATCCGAC
CAGCAAAACT CGATGTACTT CCGAGGAACT GATGTGCATA ATGCATCAGG CTGGTACATT AGATCCCCGC
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CAGTGTTGCC ACATAACCAC TATATTAACC ATTTATCTAG CGGACGCCAA AAACTCAATG TATTTCTGAG
GAAGCGTGGT GCATAATGCC ACGCAGCGTC TGCATAACTT TTATTATTTC TTTTATTAAT CAACAAAATT
TTGTTTTTAA CATTTCAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAGGGAATT CCTCGATTAA
TTAAGCGGCC GCTCGAGGGG AATTAATTCT TGAAGACGAA AGGGCCAGGT GGCACTTTTC GGGGAAATGT
GCGCGGAACC CCTATTTGTT TATTTTTCTA AATACATTCA AATATGTATC CGCTCATGAG ACAATAACCC
TGATAAATGC TTCAATAATA TTGAAAAAGG AAGAGTATGA GTATTCAACA TTTCCGTGTC GCCCTTATTC
CCTTTTTTGC GGCATTTTGC CTTCCTGTTT TTGCTCACCC AGAAACGCTG GTGAAAGTAA AAGATGCTGA
AGATCAGTTG GGTGCACGAG TGGGTTACAT CGAACTGGAT CTCAACAGCG GTAAGATCCT TGAGAGTTTT
CGCCCCGAAG AACGTTTTCC AATGATGAGC ACTTTTAAAG TTCTGCTATG TGGCGCGGTA TTATCCCGTG
TTGACGCCGG GCAAGAGCAA CTCGGTCGCC GCATACACTA TTCTCAGAAT GACTTGGTTG AGTACTCACC

AGTCACAGAA AAGCATCTTA CGGATGGCAT GACAGTAAGA GAATTATGCA GTGCTGCCAT AACCATGAGT
GATAACACTG CGGCCAACTT ACTTCTGACA ACGATCGGAG GACCGAAGGA GCTAACCGCT TTTTTGCACA
ACATGGGGGA TCATGTAACT CGCCTTGATC GTTGGGAACC GGAGCTGAAT GAAGCCATAC CAAACGACGA
GCGTGACACC ACGATGCCTG TAGCAATGGC AACAACGTTG CGCAAACTAT TAACTGGCGA ACTACTTACT
CTAGCTTCCC GGCAACAATT AATAGACTGG ATGGAGGCGG ATAAAGTTGC AGGACCACTT CTGCGCTCGG
CCCTTCCGGC TGGCTGGTTT ATTGCTGATA AATCTGGAGC CGGTGAGCGT GGGTCTCGCG GTATCATTGC
AGCACTGGGG CCAGATGGTA AGCCCTCCCG TATCGTAGTT ATCTACACGA CGGGGAGTCA GGCAACTATG
GATGAACGAA ATAGACAGAT CGCTGAGATA GGTGCCTCAC TGATTAAGCA TTGGTAACTG TCAGACCAAG
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TTTTGATAAT CTCATGACCA AAATCCCTTA ACGTGAGTTT TCGTTCCACT GAGCGTCAGA CCCCGTAGAA
AAGATCAAAG GATCTTCTTG AGATCCTTTT TTTCTGCGCG TAATCTGCTG CTTGCAAACA AAAAAACCAC
CGCTACCAGC GGTGGTTTGT TTGCCGGATC AAGAGCTACC AACTCTTTTT CCGAAGGTAA CTGGCTTCAG
CAGAGCGCAG ATACCAAATA CTGTCCTTCT AGTGTAGCCG TAGTTAGGCC ACCACTTCAA GAACTCTGTA
GCACCGCCTA CATACCTCGC TCTGCTAATC CTGTTACCAG TGGCTGCTGC CAGTGGCGAT AAGTCGTGTC
TTACCGGGTT GGACTCAAGA CGATAGTTAC CGGATAAGGC GCAGCGGTCG GGCTGAACGG GGGGTTCGTG
CACACAGCCC AGCTTGGAGC GAACGACCTA CACCGAACTG AGATACCTAC AGCGTGAGCA TTGAGAAAGC
GCCACGCTTC CCGAAGGGAG AAAGGCGGAC AGGTATCCGG TAAGCGGCAG GGTCGGAACA GGAGAGCGCA
CGAGGGAGCT TCCAGGGGGA AACGCCTGGT ATCTTTATAG TCCTGTCGGG TTTCGCCACC TCTGACTTGA
GCGTCGATTT TTGTGATGCT CGTCAGGGGG GCGGAGCCTA TGGAAAAACG CCAGCAACGC GAGCTCGTAT
GGACATATTG TCGTTAGAAC GCGGCTACAA TTAATACATA ACCTTATGTA TCATACACAT ACGATTTAGG
GGACACTATA G

The generation of SINrep5-VP22, SINrep5-E7, and SINrep5-VP22/E7 constructs have been described previously by the present inventors (Wu, TC *et al.*, co-pending International patent application published as WO01/29233 26 April 2001; see also Cheng, WF *et al.*, 2001, J Virol. *75*:2368-2376). The pcDNA3 expression vector and pcDNA3-E7 have been described (Chen, CH *et al.*, 1999, *Gene Ther 6:1972-81;* Ji, H. *et al.*, 1999, *Human Gene Therapy 10:2727-2740*). pcDNA3 has been used successfully in DNA vaccine induced immune responses and antitumor effects (Chen, CH *et al.*, 2000, *Cancer Res 60:1035-42*; co-pending, commonly assigned patent pubications or applications WO01/29233, USSN 09/421,608, filed 20 October 1999, USSN 09/501,097, filed 09 February 2000, which are incorporated by reference). For the generation of pcDNA3-VP22, VP22 was subcloned from pVP22/myc-His (Invitrogen, Carlsbad, CA) into the unique EcoRV and BamHI cloning sites of the pcDNA3.1(-) expression vector (Invitrogen, Carlsbad, CA) downstream of the CMV promoter. The generation of pcDNA3-E7 has been described previously (Chen *et al.*, *supra*). For the generation of pcDNA3-VP22/E7, VP22 was subcloned from pcDNA3-VP22 into the unique EcoRV and BamHI cloning sites of the pcDNA3-E7. For the generation of pcDNA3-E7(E/B), which contains E7 with EcoRI and BamHI restriction sites on the flanking ends of E7, PCR was used to amplify the E7 fragment with pcDNA3-E7 and a set of primers:

5′-ggggaattcatggagatacaccta-3′ (SEQ ID NO:24) and
5′-ggtggatccttgagaacagatgg-3′. (SEQ ID NO:25).

The amplified product was further cloned into the EcoRI/BamHI sites of pcDNA3. For the generation of pcDNA3-VP22(1-267)/E7, a DNA fragment encoding VP22(1-267) was first amplified using PCR with pcDNA3-VP22 and a set of primers:

5′-gggtctagaatgacctctcgccgctccgt-3′ (SEQ ID NO:26)and
5′-ggggaattcgtcctgcaccacgtctggat-3′ (SEQ ID NO:27).
The amplified product was cloned into the XbaI/EcoRI cloning sites of pcDNA3-E7(E/B).

For the generation of pcDNA3-GFP, DNA fragment encoding GFP was first amplified using PCR with pEGFPN1 DNA (Clontech, Palo Alto, CA) and a set of primers:

5′-atcggatccatggtgagcaagggcgaggag-3′ (SEQ ID NO:28)and
5′-gggaagctttacttgtacagctcgtccatg-3′. (SEQ ID NO:29)

The amplified product was cloned into the BamHI/HindIII cloning sites of pcDNA3. For the generation of pcDNA3-VP22/GFP, VP22 was subcloned from pcDNA3-VP22 into the unique EcoRV and BamHI cloning sites of the pcDNA3-GFP. For construction of pcDNA3-E7/GFP, GFP was isolated from pcDNA3-GFP and cloned into BamHI/HindIII sites of pcDNA3-E7(E+B). For construction of VP22/E7/GFP, VP22 was amplified by and a set of primers:

5′-gggtctagaatgacctctcgccgctccgt-3′ (SEQ ID NO:30) and
5′-ggggaattcctcgacgggccgtctggggc-3′ (SEQ ID NO:31)

and cloned into XbaI/EcoRI sites of pcDNA3-E7/GFP. For construction of pcDNA3-VP22(1-267)/E7/GFP, VP22(1-267) was isolated from pcDNA3-VP22(1-267) and cloned into XbaI/EcoRI sites of pcDNA3-E7/GFP.

The generation of pSC11-E7 has been described previously (Wu *et al.*, 1995, . *Proc. Natl. Acad. Sci. 92*:11671-11675). For cloning pSC11-VP22/E7, VP22 was isolated from pcDNA3-VP22/E7 by NotI/PmeI and coned into NotI/SamI sites of pSC11 vector. To generate pSC11-VP22, VP22was isolated from pcDNA3-VP22 by NotI/PmeI and cloned into NotI/SamI sites of pSC11 vector.

For the generation of pcDNA3-TAT/E7, the following complementary oligomers encoding MRKKRRQRRR (SEQ ID NO:32) (Green, M *et al.*, 1988, *Cell 55:1179-88;* Schwarze, SR *et al.*, 1999, *Science 285:1569-72*) were synthesized:

5′-ctagaatgtacggccgcaagaaacgccgccagcgccgccgcg-3′ (SEQ ID NO:33) and
5′-aattcgcggcggcgctggcggcgtttcttgcggccgtacatt-3′ (SEQ ID NO:34).
The oligomers were annealed and cloned into the XbaI/EcoRI sites of pcDNA3-E7(E/B).

For the generation of pcDNA3-E7/MTS, the following complementary oligomers encoding AAVLLPVLLAAP (SEQ ID NO:12) (Rojas, M *et al.*, 1998, *Nat Biotechnol 16:370-5*) were synthesized:

5′-gatccgcagccgttcttctccctgttcttcttgccgcacccta-3′ (SEQ ID NO:35) and
5′-agcttagggtgcggcaagaagaacagggagaagaacggctgcg-3′ (SEQ ID NO:36).

The oligomers were annealed and cloned into the BamHI/HindIII sites of pcDNA3-E7(E/B).

For the generation of pcDNA3-AH/E7, the following complementary oligomers encoding MRQIKIWFQNRRMKWKK (SEQ ID NO:15) (Derossi, D *et al.*, 1994, *J Biol Chem 269:10444-10450*) were synthesized:

5′-ctagaatgcgccaaatcaaaatctggttccagaatcgacgaatgaagtggaaaaaag-3′ (SEQ ID NO:37) and

5′-aattctttttccacttcattcgtcgattctggaaccagattttgatttggcgcatt-3′ (SEQ ID NO:38).

The oligomers were annealed and cloned into the XbaI/EcoRI sites of pcDNA3-E7(E/B). The accuracy of all the DNA constructs was confirmed by sequencing.

In Vitro RNA Preparation

The generation of RNA transcripts from SINrep5-VP22, SINrep5-E7, SINrep5-VP22/E7 and SINrep5 was performed using a protocol described previously (WO 02/09645 07-Feb-02; Cheng *et al.*, *supra*). Briefly, SpeI was used to linearize DNA templates. RNA replicons were transcribed *in vitro* and capped using SP6 RNA polymerase and capping analog from an *in vitro* transcription kit (Life Technologies, Rockville, MD) according to the vendor's manual. After synthesis, DNA was removed by digestion with DNase I. Synthesized RNA was then purified by precipitation. RNA concentration was determined by optical density measured at 260 nm. The integrity and quantity of RNA transcripts were further checked using denaturing gel electrophoresis. The purified RNA was divided into aliquots to be used for vaccination in animals and for transfection of BHK21 cells. The protein expression of the transcripts was characterized by transfection of the RNA into BHK21 cells using the Cell-Porator Electroporation System (Life Technologies, Rockville, MD) according to the vendor's manual, followed by Western blot analysis.

Generation of SINrep5 Replicon Particles and Determination of the Vector Titer

SINrep5 replicon particles were made using a protocol described protocol by Polo *et al.* *supra*. Briefly, 4 μg of mRNA synthesized *in vitro* was electroporated into 10^7 cells of the PCL. The PCL cells were incubated in 23 ml DMEM supplemented with 10% FBS, antibiotics and G418 at 5% CO_2, 37°C. After 72 hr, culture supernatants were collected. The titer of SINrep5

replicon particles in clarified PCL culture supernatants was determined by infection of naïve BHK-21 monolayers, followed by indirect E7 immunofluorescence staining (Wu *et al.*, 1995, . *Proc. Natl. Acad. Sci. 92*:11671-11675) with serial dilution and quantitation of the total number of green stained cells per well at each dilution. Vector titer is designated as infectious units (IU)/ml, and represents the population of functional particles.

Immunofluorescence Staining for E7 and VP22/E7 Expression

Immunofluorescence staining was performed as described by (Wu *et al.*, *supra*). Briefly, BHK21 cells were cultured in 2-well culture chamber slides (Nalge Nunc Int., Naperville, IL) until they reached 50% confluency. The BHK21 cells were infected with a serial dilution of replicon particles. After 48 and 72 hours of infection, the cells were fixed in 10% formalin for 20 min. Diluted anti-E7 Ab (1: 200 dilution, Zymed, San Francisco, CA) was added into the chamber and incubated for 30 min. Diluted FITC goat anti-mouse IgG (10 μg/ml, Jackson ImmunoReseach Laboratories, West Grove, PA), was added and incubated for 30 min. The slides were mounted and observed immediately under a fluorescence microscope.

Mice

6- to 8-week-old female C57BL/6 mice from the National Cancer Institute (Frederick, MD) were purchased and kept in the oncology animal facility of the Johns Hopkins Hospital (Baltimore, MD). All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals.

Intracytoplasmic Cytokine Staining and Flow Cytometry Analysis

Intracytoplasmic cytokine staining and flow cytometry analysis were performed as described previously (Cheng *et al.*, 2001b, Human Gene Therapy. *12*:235-252). In brief, mice (5 per group) were vaccinated with $5x10^6$ IU/mouse of SINrep5-VP22/E7 via different routes of administration (intramuscular, intraperitoneal, subcutaneous). In another experiment, another group of mice (5 per group) was vaccinated intramuscularly with different titers of SINrep5 replicon particles ($5x10^7$, $5x10^6$, $5x10^5$ IU $5x10^4$, and $5x10^3$ IU/mouse). Naïve mice served as negative controls. Splenocytes from vaccinated mice were collected seven days after vaccination and incubated either with the E7 peptide (aa 49-57, RAHYNIVTF) containing the MHC class I epitope (Feltkamp *et al.*, 1993, Eur J Immunol. *23*:2242-2249) (to detect E7-specific $CD8^+$ cytotoxic T cell precursors) or with the E7 peptide (aa 30-67, DSSEEEDEIDGPAGQAEPDRAHYNIVTFCCKCDSTLRL; SEQ ID NO:39) that includes the

MHC class II epitope (Tindle *et al.*, 1991, Proc Natl Acad Sci USA *88*:5887-5891) (to detect E7-specific $CD4^+$ T helper cell precursors) for 20 hours. Cells were subjected to intracellular cytokine staining using the Cytofix/Cytoperm kit according to the manufacturer's instructions (PharMingen, San Diego, CA). Analysis was done on a Becton Dickinson FACScan with CELLQuest software (Becton Dickinson Immunocytometry System, Mountain View, CA).

ELISA

Anti-HPV 16 E7 antibodies in the sera from vaccinated mice (5 per group) were determined by a direct ELISA as described previously (Wu *et al.*, *supra*). The ELISA plate was read with a standard ELISA reader at 450 nm.

The quantity of E7 protein in cell lysates from BHK21 cells infected with SIN5rep-E7 or SINrep5-VP22/E7 replicon particles was determined by an indirect ELISA method as described previously (Cheng *et al.*, *supra*). Briefly, $1x10^7$ BHK21 cells were infected with $5x10^7$ IU of SINrep5, SINrep5-E7, SINrep5-VP22 or SINrep5-VP22/E7 particles. The transfected BHK21 cells were collected 40-44 hrs after infection. The quantity of E7 protein in cell lysates from transfected BHK21 cells was determined by ELISA with a standard ELISA reader at 450 nm. The quantity of E7 protein of cell lysates was then calculated and determined by comparing with the standardized E7 protein.

***In Vivo* Tumor Protection**

For the tumor protection experiment, mice (5 per group) were immunized intramuscularly with $5x10^6$ IU/mouse of SINrep5-E7, SINrep5-VP22, SINrep5-VP22/E7 or control SINrep5 replicon particles. One week after vaccination, mice were subcutaneously challenged with 1 x 10^4 TC-1 cells/mouse in the right leg. Mice were monitored for evidence of tumor growth by palpation and inspection twice a week until they were sacrificed at day 60.

***In Vivo* Tumor Therapy**

C57BL/6 mice (5 each group) were challenged with 10^4 cells/mouse TC-1 tumor cells i.v. in the tail vein on day 0. Three, seven or fourteen days after tumor challenge, mice were treated intramuscularly with $5x10^6$ IU/mouse of SINrep5-E7, SINrep5-VP22, SINrep5-VP22/E7 or SINrep5 control replicon particles. Mice were sacrificed on day 21 after tumor challenge. The number of pulmonary tumor nodules on the surface of lungs in each mouse was determined by experimenters blinded to the sample identity.

For the head-to-head comparison of various VP22/E7-containing vaccines, we performed another in vivo tumor treatment experiment. C57BL/6 mice (5 each group) were intravenously challenged with 10^4 cells/mouse TC-1 tumor cells in the tail vein on day 0. 7 days after tumor challenge, mice were treated intramuscularly with optimized vaccine doses determined from previous studies: 2 μg/mouse SINrep5-VP22/E7 DNA (Hung *et al.*, 2001, *supra*), 1 μg/mouse SINrep5-VP22/E7 RNA (Cheng *et al.*, 2001a), or $5x10^6$ IU/mouse of SINrep5-VP22/E7 replicon particles. Naïve mice were used as a negative control. Mice were sacrificed on day 28 after tumor challenge and mean lung weight was measured by experimenters blinded to the sample identity.

***In Vivo* Antibody Depletion Experiments**

The procedure for *in vivo* antibody depletion has been described previously. Briefly, mice were vaccinated intramuscularly with $5x10^6$ IU/mouse of SINrep5-VP22/E7 replicon particles. Depletions were started on day 7 after immunization and mice were challenged with 1×10^4 cells/mouse TC-1 tumor cells on day 14 after immunization. MAb GK1.5 (Dialynas *et al.*, 1983, J. Immunol. J. Immunol. *131*:2445:2445) was used for CD4 depletion, MAb 2.43 (Sarmiento *et al.*, 1980, J. Immunol. *125*:2665) was used for CD8 depletion, and MAb PK136 (Koo *et al.*, 1986, J. Immunol. *137*:3742) was used for NK1.1 depletion. Flow cytometry analysis revealed that >99% of the appropriate lymphocyte subsets were depleted while maintaining normal levels of other subsets. Depletion was terminated on day 40 after tumor challenge.

***In Vivo* Assay for Apoptotic Cells in the Tissue of Vaccinated Mice**

Mice were immunized with $5x10^6$ IU/mouse of SINrep5-VP22/E7 replicon particles intramuscularly in the right leg. Normal saline without replicon particles was injected intramuscularly into the left leg as a control. Mice were sacrificed 7 days after intramuscular injection. For the detection of apoptotic cells, a modified TUNEL method was used as described previously (Cheng *et al.*, *supra*). Apoptotic index is used as a measure of the extent of apoptosis in the stained slides following inspection under a light microscope. Apoptotic index is defined as the percentage of apoptotic cells and apoptotic bodies per 100 cells (Lipponen *et al.*, 1994, J Pathol. **173**:333-339).

CTL Assay Using DCs Co-Incubated with SIN Replicon-Infected BHK21 Cells

DCs were generated by culturing bone marrow cells in the presence of GM-CSF as described previously (Cheng *et al.*, *Human Gene Ther.*, 2001). CTL assays, using DCs co-

incubated with transfected cells as targets, were performed using a previously described protocol (Cheng *et al.*, *supra*). Briefly, 10^7 BHK21 cells were infected with $5x10^7$ IU of SINrep5, SINrep5-E7, SINrep5-VP22 or SINrep5-VP22/E7 replicon particles. The infected BHK21 cells were collected 40-44 hrs later. The levels of E7 protein expression for all replicon-infected BHK21 cells were similar, as determined by ELISA. $3x10^5$ of infected BHK21 cells were then co-incubated with $1x10^5$ of bone marrow-derived DCs at 37°C for 48 hr. These prepared DCs were then used as target cells and an E7-specific $CD8^+$ T cell line (Wang *et al.*, 2000, *supra*) served as effector cells. CTL assays were performed with effector cells and target cells ($1x10^4$ per well) mixed together at various ratios (1:1, 3:1, 9:1, and 27:1) in a final volume of 200 µl. After a 5 hr incubation at 37°C, 50 µl of the cultured media was collected to assess the amount of LDH using the CytoTox assay kit (Promega, Madison, WI). The percentage of lysis was calculated from the formula:

$$\% \text{ Lysis} = [(A-B)/(C-D)] \times 100$$

where A is the experimental-effector signal value, B is the effector spontaneous background signal value, C is maximum signal value from target cells, D is the target spontaneous background signal value. DCs co-incubated with uninfected BHK21 cells, infected BHK21 cells alone, untreated DCs alone, and $CD8^+$ T cells alone were included as negative controls.

Fluorescence Microscopy for *In vitro* Distribution of VP22/E7

293 D^bK^b cells (provided by Dr. JC Yang, National Cancer Institute, NIH; Bloom, MB *et al.*, 1997, *J Exp Med 185:453-459*) were utilized for an *in vitro* assay of GFP expression. 20µg of VP22, E7/GFP, VP22(1-267)/E7/GFP or VP22/E7/GFP DNA were transfected into $5x10^6$ 293 D^bK^b cells using lipofectamine 2000 (Life Technologies, Rockville, MD). Transfected cells were fixed with 4 % paraformaldehyde in 1X PBS, permeabilized with 1X PBS containing 0.05% saponin and 1% BSA, then incubated with 0.5 µg/ml of primary anti-calnexin antibody (Stressgen Biotechnologies, Victoria, BC). Samples were acquired with the Noran Oz confocal laser scanning microscope system using Invertension® software (v. 6.5). Slides were imaged with an Olympus IX-50 inverted microscope (100x magnification).

Immunohistochemical Staining for In Vivo Distribution of VP22/E7

Mice were sacrificed 3 days after vaccination with pcDNA3-VP22/GFP or pcDNA3-. Skin was biopsied, fixed, paraffin-embedded, and cut into 5 µm sections. After deparaffinization

and hydration, slides were incubated with rabbit anti-GFP polyclonal antibody (1:200 dilution; Molecular Probes, Eugene, OR) followed by biotinylated goat anti-rabbit IgG (1:200 dilution) and avidin-biotin complex (1:100 dilution; Vector, Burlingame, CA). The slides were developed by adding DAB substrate solution (DAKO, Carpenteria, CA) and counterstained with Mayer's hematoxylin. Stained slides were dehydrated, mounted and observed by light microscopy.

EXAMPLE II

Expression and Distribution of E7 and Chimeric VP22/E7 Protein in Cells Infected with SINrep5-E7 or SINrep5-VP22/E7 Replicon Particles

To demonstrate the expression of E7 protein in cells infected with E7-encoding SINrep5 replicon particles, indirect ELISA and immunofluorescence staining were performed. The quantity of E7 protein was determined using lysates from cells infected with $5x10^7$ IU of SINrep5, SINrep5-E7, SINrep5-VP22 or SINrep5-VP22/E7 replicon particles.

The level of E7 and VP22/E7 protein expression was comparable between cells infected with SINrep5-E7 and SINrep5–VP22/E7 replicon particles 48 hours after infection.

The subcellular localization of E7 and VP22/E7 proteins in the infected BHK21 cells was also evaluated by immunofluorescence 48 hours after infection. E7 protein was mainly located in the nucleus (**Figure 1A**), while chimeric VP22/E7 protein was located in the cytoplasm (**Figure 1B**). 72 hours after infection, E7 protein remained localized predominantly in the nucleus of the initially infected BHK21 cells (**Figure 1C**). Meanwhile, chimeric VP22/E7 protein was transferred to cells neighboring the initially infected BHK21 cells 72 hours after infection (**Figure 1D**).

The results indicated that the linkage of VP22 to antigen in SINrep5 replicon particles led to the intercellular spread of linked antigen, which was observable at a later stage of infection (72 hours after infection).

EXAMPLE III

SINrep5-VP22/E7 Replicon Particles Significantly Enhance E7-Specific $CD8^+$ T Cell Responses but not $CD4^+$ Cell-Mediated Immune Responses

To determine the quantity of E7-specific $CD8^+$ T cell precursors in mice vaccinated with SINrep5-VP22/E7 replicon particles, intracellular cytokine staining was performed as described above. As shown in **Figure 2A**, mice vaccinated with SINrep5-VP22/E7 replicon particles generated the greatest number of E7-specific $CD8^+$ T cell precursors compared to the other

vaccination groups ($p<0.001$). SINrep5-VP22/E7 replicon particles generated a significant 18-fold increase in the number of E7-specific $CD8^+$ T cell precursors compared to that generated by wild-type E7 expressing particles (219 ± 12.7 versus 13.5 ± 2.1 per $3x10^5$ splenocytes, $p<0.001$) (**Figure 2B**).

In comparison, the number of E7-specific $CD4^+$ T cell precursors generated by the different SIN replicon particles were not significantly different (**Figure 2C**).

Anti-E7 antibody titers generated by SINrep5-VP22/E7 replicon particles were not increased compared to the other groups. These results were consistent with the observed lack of E7-specific $CD4^+$ T helper cell enhancement.

Fusion of E7 to VP22 was required for enhanced $CD8^+$ T cell activity, since VP22 mixed with E7 (VP22 + E7) did not cause such enhancement of $CD8^+$ T cell activity. Furthermore, E7 linked to an irrelevant protein such as green fluorescent protein (GFP) did not enhance E7-specific $CD8^+$ T cell activity.

EXAMPLE IV

Different Routes and Doses of SINrep5-VP22/E7 Replicon Particles Influence the Antigen-Specific $CD8^+$ T Cell-mediated Response

The relationship between different routes of vaccine administration and the resultig immune responses were evaluated. Mice were injected i.m., i.p, and s.c., with $5x10^6$ IU/mouse of SINrep5-VP22/E7 replicon particles. Intramuscular injection generated more E7-specific $CD8^+$ T cells than the other two routes (**Figure 3A**). The correlation between replicon particle dosages and responses were also evaluated. Different doses of SINrep5-VP22/E7 replicon particles were injected i.m. into mice. With increasing doses of replicon particles, the number of E7-specific $CD8^+$ T cell precursors progressively increased until reaching a plateau at the dose of $5x10^6$ IU/mouse (**Figure 3B**). These results suggested that i.m. delivery of SINrep5-VP22/E7 replicon particles at a dose of $5x10^6$ IU/mouse was optimal for generating an E7-specific CD8+ T cell immune response.

EXAMPLE V

Antitumor Effects of SINrep5-VP22/E7 Replicon Particle Vaccine: Tumor Protection

To determine whether vaccination with the various SIN replicon particles protected mice against E7-expressing tumors, *in vivo* tumor protection experiments were performed as described

in Example I. Figure 4 shows that all mice receiving SINrep5-VP22/E7 particles remained tumor-free 60 days after TC-1 challenge. In contrast, all of the unvaccinated mice and mice receiving SINrep5 with no insert, VP22, wild-type E7, or VP22 + E7 developed visible tumors within 20 days after tumor challenge.

These results also indicated that fusion of E7 to VP22 was required for antitumor immunity since VP22 mixed with E7 (VP22 + E7) did not improve the antitumor effect.

EXAMPLE VI

Anitutmor Effects of SINrep5-VP22/E7 Replicon Particles: Eradication of Established Lung Tumors in the Lungs

To determine the therapeutic potential of the SINrep5-VP22/E7 vaccine, C57BL/6 mice were challenged i.v. with 10^4 TC-1 tumor cells per mouse in the tail vein to establish TC-1 tumors in the lungs (Ji *et al.*, 1998, Int J Cancer. 78:41-45). This model permits a more quantitative assessment of antitumor effects than the subcutaneous model. **Figure 5A** demonstrated that mice treated with SINrep5-VP22/E7 replicon particles three days after tumor challenge exhibited a significantly lower mean number of pulmonary nodules (0.7$\pm$ 0.3) than mice vaccinated with wild-type E7 replicon particles (72.5$\pm$ 8.5) or VP22 replicon particles (79.0$\pm$17.0). As shown in **Figure 5B**, mice treated with SINrep5-VP22/E7 replicon particles displayed a significantly fewer pulmonary nodules on day 3 (0.7$\pm$0.3), day 7 (0.5$\pm$0.3) and day 14 (25.0$\pm$4.0) after tumor challenge compared to mice treated with the SINrep5 control particles (no insert)(one-way ANOVA, $p < 0.05$). Treatment with SINrep5-VP22/E7 replicon particles reduced grossly visible tumors even if administered as late as 14 days after tumor challenge. In general, grossly visible lung nodules could be detected 14 days after i.v. tumor (TC-1) cell injection. The results indicated that SINrep5-VP22/E7 particles can control established life-threatening tumor growth, even up to 14 days after the tumor has been implanted.

EXAMPLE VII

Sindbis Virus Replicon Particles Are the Most Effective Delivery Vector for VP22/E7 Vaccines used to Controlling Established Pulmonary Tumors

Previously, the prseent inventors and their colleagues developed a DNA vaccine (Hung *et al.*, 2001, *supra*) and naked RNA replicon vaccine (Cheng *et al.*, 2001a, *supra* ; Wu *et al.*, WO02/09645) encoding VP22/E7 and found that they were effective for treatment of TC-1 tumor cells.

To compare the relative efficacy of VP22/E7 naked DNA, naked SINrep5-VP22/E7 RNA replicons, and SINrep5-VP22/E7 RNA replicon particles, C57BL/6 mice were challenged with 10^4 TC-1 tumor cells per mouse i.v. via the tail vein to establish pulmonary TC-1 tumors, followed by treatment with optimized doses of the various VP22/E7-containing vaccines 7 days later. **Figure 6A** demonstrated that mice treated with SINrep5-VP22/E7 replicon particles exhibited a significantly lower mean lung weight (208±13 mg) than did mice vaccinated with VP22/E7 naked DNA (256±33 mg) or naked SINrep5-VP22/E7 RNA replicons (521±53 mg), or naïve mice (644±44 mg). **Figure 6B** displays representative gross pictures of pulmonary metastatic nodules derived from mice treated with different VP22/E7-containing vaccines. These results indicated that treatment of mice with SINrep5-VP22/E7 replicon particles generated the greatest therapeutic antitumor effect among the VP22/E7 chimeric vaccines.

EXAMPLE VIII

CD8$^+$ T cells, CD4$^+$ T cells and NK cells are Essential for the Anti-tumor Effect Generated by SINrep5-VP22/E7 Replicon Particles

To determine which subset(s) of lymphocytes that are important for the rejection of E7+ tumor cells, *in vivo* antibody depletion experiments were done. The completeness of depletion was assessed on the day of tumor injection and weekly thereafter by flow cytometric analysis of spleen cells. Typically, the appropriate subset was depeleted by > 99% while other lymphocytes populations remained at normal levels. As shown in Figure 7, tumors grew in all naïve mice and all mice depleted of CD8$^+$ T cells within 14 days after implantation. 80% of mice depleted of CD4$^+$ T cells and 60% of mice depleted of NK1.1 cells developed tumors within 60 days of implantation. These results suggested that CD8$^+$ T cells, CD4$^+$ T cells and NK cells all participate in the anti-tumor immunity induced by the SINrep5-VP22/E7 replicon particles.

EXAMPLE IX

Apoptotic in Cells Infected with SIN Replicon Particles in Vaccinated Mice

To evaluate whether SIN replicon particles induce apoptosis *in vivo*, mice were immunized i.m. with $5x10^6$ IU each of SINrep5-VP22/E7 replicon particles and were sacrificed 7 days later. Tissue sections of muscle were stained using the TUNEL method described above. Cells undergoing apoptosis exhibited brown staining of nuclei. As shown in Figure 8, vaccination

with SIN replicon vaccines led to a significantly higher number of apoptotic cells in muscle tissue as compared to tisse from mice given normal saline. The apoptotic index was 74.5±4.5 for the SINrep5-VP22/E7-treated group and 30.5±2.5 for the saline control ($p<0.01$). These results indicated that muscle cells infected with SIN replicon particles underwent apoptosis.

EXAMPLE X

Enhanced Presentation of E7 Through the MHC Class I Pathway in Dendritic Cells Pulsed With Lysate of Cells Infected by SINrep5-VP22/E7 Replicon Particles

Of the various treatments, vaccination with SINrep5-VP22/E7 replicon particles resulted in the most potent immune response measured as the number of E7-specific $CD8^+$ T cell precursors (**Figure 3A**). One mechanism for enhanced E7-specific $CD8^+$ T cell responses *in vivo* is presentation of E7 through the MHC class I pathway by APCs that have taken up apoptotic cells in whichthe various antigen constructs were expressed. This phenomenon is known as "cross-priming".

To determine whether SINrep5-VP22/E7 particles induce a cell-mediated immune response via cross-priming, BHK21 cells were first infected with various antigen-containing and control SIN replicon particles. These infected BHK21 cells were then incubated with bone marrow-derived DCs and used as target cells. Cytotoxic effector cells were T cells of an E7-specific $CD8^+$ T cell line. As shown in Figure 9, the E7-specific CTL lysed DCs that had been "pulsed" with BHK21 cells infected with SINrep5-VP22/E7 more effectively than they lysed DCs pulsed with BHK21 cells that had been infected with other replicon particles (at E:T ratios of 9 and 27 ($p<0.01$). Thus, DCs "pulsed" with SINrep5-VP22/E7 infected cells present E7 antigen through the MHC class I pathway more efficiently than do DCs pulsed with SINrep5-E7 infected cells.

DISCUSSION OF EXAMPLES I-X

As described above, the present inventors generated SIN replicon vectors from a stable PCL for vaccine development. The use of a stable PCL allowed the production of high titers of SIN replicon particles free of replication-competent virus, representing an important advance in the preparation of vaccines for mass immunization. Although SIN infection in humans typically has limited clinical manifestations, fever, skin rash, and arthritic joint pain have been reported in people infected with certain Sindbis virus strains (Strauss & Strauss, *supra*). The separation of structural protein cassettes in alphavirus PCLs significantly decreases the possibility of

producing replication-competent virus (Polo *et al., supra*) and therefore decreases the likelihood of such undesired clinical effects.

An important consideration for vaccine development is the titer of vector stocks produced from this or other stable PCL. As shown above, the present inventors successfully produced SIN replicon particle stocks with titers up to $5x10^7$ IU/ml, which was slightly higher than that described by Polo *et al., supra*. Wild-type Sindbis virus infection is known to generate titers of greater than 10^9 PFU/ml. Research has focused on improving transport between the nucleus and cytoplasm as well as stabilizing primary alphavirus RNA transcripts (Polo *et al., supra*), two approaches that would contribute to higher titers of the replicon vectors approaching the level of wild-type alphavirus. Indeed, a panel of recently constructed SIN replicon PCL consistently produced particle titers of $>0^8$ IU/ml (??PFU) (C. Greer, B. Belli, and J. Polo unpublished data).

One limitation of such replication-defective viral vectors, whicha re relatively safe, one is their intrinsic inability to spread *in vivo* as effectively as do replication-competent viruses . As described herein, the present invention's inclusion of an intercellular spreading protein, exemplified as HSV-1VP22, fused to antigen in the context of SIN replicon vectors, facilitated the spread of antigen to surrounding cells *in vivo* resulting in a significantly enhanced E7-specific $CD8^+$ T cell response and consequent antitumor effects. Thus, the strategy of using a an intercellular spreading protein fused to an antigen and producing the vectors containing the nucleic acid expressing this fusion polypeptide by employing a stable PCL represents a unique and novel approach for generating a safe, potent vaccine in high quantities. This strategy provides several advantages over other vaccine approaches. Compared to naked nucleic acid vaccines, SIN replicon particles are capable of infecting/transfecting a higher proportion of "target" cells. The linkage of the intercellular spreading protein, *e.g.*, HSV VP22, further enhances vaccine potency. Another advantage is that SIN replicon RNA does not integrate into the host genome, which is a potential concern with naked DNA vaccines or DNA-based viral vectors. The composition comprising SIN replicon vectors that is generated from a stable PCL such as that exemplifed here is free of replication-competent virus particles without sacrificing the efficiency of gene delivery. This feature maximizes vaccine potency while minimizing the risk associated with replication-competent viral vectors. Finally, stable PCLs are also versatile, allowing for the packaging of different alphavirus-derived replicon vectors, in the present example, either Sindbis or Semliki Forest virus derived replicon vectors.

As disclosed above, treatment of mice with SINrep5-VP22/E7 replicon particles led to a more potent antitumor effect than did treatment with VP22/E7 naked DNA or naked SINrep5-VP22/E7 RNA replicon vaccines. It is noteworthy that that in mice vaccinated with VP22/E7 DNA (as reported elsewhere), a higher frequency of antigen-specific CD8+ T cell precursors were detected ($576/3x10^5$ splenocytes) (Hung *et al.*, 2001, *supra*) compared to the present examples of mice vaccinated with SINrep5-VP22/E7 replicon particles ($219/3x10^5$ splenocytes, **Figure 2B**) at one week after the final vaccination. One explanation for this difference is a difference in the kinetics of generation of antigen-specific T cells by the various types of vaccines. Thus, vaccination with a Sindbis virus replicon particle vaccine resulted in peak numbers of antigen-specific CD8+ T cells earlier than vaccination with a DNA vaccine (3 days vs. 11 days. Thus, the rapid expansion of antigen-specific T cells after vaccination with Sindbis virus replicon particles is expected to exert more effective responses against rapidly growing tumors than would the relatively slower expansion of antigen-specific T cells following DNA vaccination.

The present inventors tested the strategy of combining an intercellular spreading protein, such as HSV-1 VP22, with antigen while comparing different delivery vectors: naked DNA (pcDNA3) and naked SIN RNA (SINrep5). Each vaccine enhanced E7-specific $CD8^+$ T cell-mediated immune responses and antitumor effects, although the effector cell involvement was different. $CD8^+$ T cells were important components of the responses to all of the vectors tested, while $CD4^+$ T cells were only essential for the antitumor effect generated by the VP22/E7 SIN particle-based vaccine. This conclusion is based on the observation that depleting $CD4^+$ T cells did not diminish antitumor effects of the naked DNA vaccine -- pcDNA3-VP22/E7 (Hung *et al.*, 2001, *supra*) or the naked SIN replicon RNA vaccine -- SIN replicon RNA-VP22/E7 (Cheng *et al.*, 2001, *J. Virol., supra*). Although $CD4^+$ T cells appeared to be needed for an optimal antitumor effect in response to the VP22/E7 SIN particle-based vaccine described herein, this vaccine did not actively induce E7-specific CD4+ T cells. This suggested that these $CD4^+$ T cells were contributing to an antitumor effect via a non-antigen-specific mechanism. Indeed, NK cells were needed for the present antitumor effect but were not as important in response to the VP22/E7 SIN particle-based vaccine or the naked DNA vaccine. Thus, different types of vaccines encoding the same protein construct may activate different subsets of effector cells in the vaccinated host and activate different immune or nonimmune antitumor mechanisms.

The enhanced E7-specific $CD8^+$ T cell responses induced by the present VP22/E7 SIN replicon particle vaccine compared to a "control" E7 SIN replicon particle vaccine are believed

to result, at least in part, from a process whereby infectected apoptotic cells are endocytosed and processed by APCs for MHC class I antigen presentation to $CD8^+$ T cells (Albert, ML *et al.*, 1998, *J Exp Med. 188*:1359-1368.; Albert, ML *et al.*, 1998, *Nature. 392*:86-89). Alternatively, apoptotic cells may release chimeric VP22/E7 proteins that are taken up and processed by other APCs via a MHC class I-restricted pathway (Huang *et al.*, *supra*). Because different types of SINrep5 replicon particles induced similar degrees of apoptosis, the distinct enhancement in E7-specific $CD8^+$ T cell activity was interpreted by the presen inventors as most likely due to the linkage of VP22 with E7. It is unlikely that the observed enhancement occurs as a result of improved direct MHC class I presentation of E7 to CTLs by cells expressing VP22/E7 because the replicon-infected cells eventually undergo apoptosis.

Recently, Gardner *et al.* (2000, *J. Virol. 74*:11849-11857) reported that SIN replicon particles encoding a modified E2 glycoprotein successfully delivered genes of interest into DCs to create a DC -based vaccine that could induce potent immune responses in vaccinated mice. DCs are the most potent APC and play a major role in the activation of both memory and naïve T cells. Therefore, as conceived herein, the employment of SIN replicon particles for preparing effective DC compositions is a significant extension of the heretofore dislcosed uses of these recombinant alphaviral vectors. Thus, it is expected that the combination of intercellular spreading protein fusion strategy with the convenience of generating SIN replicon particles from stable PCLs will improve the efficiency of antigen delivery into DCs and permit development of improved DC -based immunotherapeutic vaccines.

In summary, these results revealed that the combined usage of the viral spreading protein strategy along with an efficient method of producing safe SIN replicon particle preparations free of replication-competent virus was effective in generating potent antigen-specific immune responses and a strong antitumor effect. Furthermore, the availability of this stable alphavirus PCL makes it possible to generate a large quantity of the replicon particle-based vaccine for mass immunization. These strategies may also be applied to other cancer systems and infectious

WHAT IS CLAIMED IS

1. A nucleic acid molecule encoding a fusion polypeptide useful as a vaccine composition, which molecule comprises:

(a) a first nucleic acid sequence encoding a first polypeptide that comprises at least one immunogenicity-potentiating polypeptide;

(b) optionally, fused in frame with the first nucleic acid sequence, a linker nucleic acid sequence encoding a linker peptide; and

(c) a second nucleic acid sequence that is linked in frame to said first nucleic acid sequence or to said linker nucleic acid sequence and that encodes an antigenic polypeptide or peptide,

which nucleic acid is in the form of a replication-defective alphavirus replicon particle prepared using a packaging cell line.

2. The nucleic acid molecule of claim 1 wherein the first polypeptide is one that acts by promoting:

(a) processing of the linked antigenic polypeptide via the MHC class I pathway or targeting of a cellular compartment that increases said processing;

(b) development, accumulation or activity of antigen presenting cells or targeting of antigen to compartments of said antigen presenting cells leading to enhanced antigen presentation;

(c) intercellular transport and spreading of the antigen; or

(d) any combination of (a)-(c).

3. The nucleic acid molecule of claim 1 or 2 wherein the first polypeptide is:

(a) a mycobacterial HSP70 polypeptide, the C-terminal domain thereof, or a functional homologue or derivative of said polypeptide or domain;

(b) a viral intercellular spreading protein selected from the group of herpes simplex virus-1 VP22 protein, Marek's disease virus VP22 protein or a functional homologue or derivative thereof;

(c) an endoplasmic reticulum chaperone polypeptide selected from the group of calreticulin, ER60, GRP94, gp96, or a functional homologue or derivative thereof

(d) a cytoplasmic translocation polypeptide domains of a pathogen toxin selected from the group of domain II of *Pseudomonas* exotoxin ETA (ETAdII) or a functional homologue or derivative thereof;

(e) a polypeptide that targets the centrosome compartment of a cell selected from γ-tubulin or a functional homologue or derivative thereof; or

(f) a polypeptide that stimulates dendritic cell processors or activates dendritic cell activity selected from the group of GM-CSF, Flt3-ligand extracellular domain, or a functional homologue or derivative thereof

4. nucleic acid molecule of any of claims 1-3 wherein the first polypeptide is selected from the group consisting of *Mycobacterium tuberculosis* HSP70, the HSP70 C-terminal domain, HSV-1 VP22, MDV VP22, calreticulin, *Pseudomonas* ETAdII, GM-CSF, Flt-3 ligand extracellular domain or γ-tubulin.

5. The nucleic acid molecule of claim wherein the first polypeptide is a transport polypeptide comprising SEQ ID NO:5 or 7 or an active fraagment thereof.

6. The nucleic acid molecule of any of claims 1-4 wherein the antigenic polypeptide comprises an epitope that binds to, and is presented on the cell surface by, an MHC class I protein.

7. The nucleic acid molecule of claim 5, wherein said epitope is between about 8 and about 11 amino acid residues in length.

8. The nucleic acid molecule of any of claims 1-8 wherein the antigen is one which is present on, or cross-reactive with an epitope of, a pathogenic organism, cell, or virus.

9. The nucleic acid molecule of claim 8, wherein the virus is a human papilloma virus.

10. The nucleic acid molecule of claim 9, wherein the antigen is the E7 polypeptide of HPV-16 or an antigenic fragment thereof.

11. The nucleic acid molecule of claim 8, wherein the pathogenic organism is a bacterium.

12. The nucleic acid molecule of claim 8, wherein the pathogenic cell is a tumor cell.

13. The nucleic acid molecule of claim 12, wherein the antigen is a tumor-specific or tumor-associated antigen.

14. The nucleic acid molecule of claim 13, wherein the antigen comprises a peptide of the HER-2/neu protein.

15. The nucleic acid molecule of any of claims 1-14 operatively linked to a promoter.

16. The nucleic acid molecule of claim 15 , wherein the promoter is one which is expressed in an antigen presenting cell (APC).

17. The nucleic acid molecule of claim 16, wherein the APC is a dendritic cell.

18. The nucleic acid molecule of any of claims 1-17 wherein the alphavirus is Sindbis virus, Semliki forest virus or Venezuelan equine encephalitis virus.

19. The nucleic acid molecule of claim 18 wherein the alphavirus is Sindbis virus.

20. The nucleic acid molecule of claim 19 wherein the Sindbis virus replicon is SINrep5.

21. The nucleic acid molecule of any of claims 1-20 wherein the packaging cell line is one in which genes encoding capsid and envelope glycoproteins of said alphavirus are separated in distinct cassettes to minimize formation of replication competent virus during replicon production.

22. The nucleic acid molecule of any of claims 18-21 wherein the packaging cell line is 987dlsplit #24.

23. An expression vector comprising the nucleic acid molecule of any of claims 1-22 operatively linked to

(a) a promoter; and

(b) optionally, additional regulatory sequences that regulate expression of said nucleic acid in a eukaryotic cell.

24. A cell which has been modified to comprise the nucleic acid or expression vector of any of claims 1-23.

25. The cell of claim 31 which expresses said nucleic acid molecule.

26. The cell of claim 24 or 25 which is an APC.

27. The cell of claim 26, wherein the APC is a dendritic cell, a keratinocyte, a macrophage, a monocyte, a B lymphocyte, a microglial cell, an astrocyte, or an activated endothelial cell.

28. A pharmaceutical composition capable of inducing or enhancing an antigen-specific immune response, comprising:

(a) pharmaceutically and immunologically acceptable excipient in combination with;

(b) a composition selected from the group consisting of:

(i) the nucleic acid molecule or expression vector of any of claims 1-23;

(ii) the cell of any of claims 24-27

(iii) any combination of (i) and (ii).

29. A method of inducing or enhancing an antigen specific immune response in a subject comprising administering to the subject an effective amount of the pharmaceutical composition of claim 28, thereby inducing or enhancing said response.

30. The method of claim 28 or 29, wherein the response is mediated at least in part by $CD8^+$ cytotoxic T lymphocytes (CTL).

31. The method of claim 28 or 29, wherein the response is mediated at least in part by antibodies.

32. A method of inducing or enhancing an antigen specific immune response in cells or in a subject comprising administering to said cells or to said subject an effective amount of the pharmaceutical composition of claim 28, thereby inducing or enhancing said response.

33. The method of claim 32, wherein the composition is administered *ex vivo* to said cells.

34. The method of claim 32 wherein said cells comprise APCs.

35. The method of claim 54, wherein said APCs are dendritic cells.

36. The method of claim 34 or 35, wherein the APCs are human APCs.

37. The method of any of claims 34-36, wherein the APCs are isolated from a living subject.

38. The method of any of claims 32-37, further comprising a step of administering the *ex vivo*-treated cells to a histocompatible subject.

39. The method of any of claims 29-38 wherein said cells are human cells and said subject is a human.

40. The method of any of claims 29-32, 38 and 39 wherein said administering is by a intramuscular, intradermal, or subcutaneous route.

41. The method of any of claims 29-32 and 38-40 wherein the administering is intratumoral or peritumoral.

42. A method of increasing the numbers or lytic activity of $CD8^+$ CTLs specific for a selected antigen in a subject, comprising administering to said subject an effective amount of a composition selected from the group consisting of:

(a) the nucleic acid molecule or expression vector of any of claims 1-23;

(b) the cell of any of claims 24-27

(c) any combination of (a) and (b).

wherein

(i) said nucleic acid molecule, said expression vector or said cell comprises said antigen,

(i) said antigen comprises an epitope that binds to, and is presented on the cell surface by, MHC class I proteins,

thereby increasing the numbers or activity of said CTLs.

43. A method of inhibiting growth or preventing re-growth of a tumor in a subject, comprising administering to said subject an effective amount of a composition selected from the group consisting of:

(a) the nucleic acid molecule or expression vector of any of claims 1-23;

(b) the cell of any of claims 24-27; and

(c) any combination of (a) and (b).

wherein

(i) said nucleic acid molecule, said expression vector or said cell comprises said antigen,

(ii) said antigen comprises one or more tumor-associated or tumor-specific epitopes present on said tumor in said subject

thereby inhibiting said growth or preventing said re-growth.

44. The method of claim 43, wherein said administering is intratumoral or peritumoral.

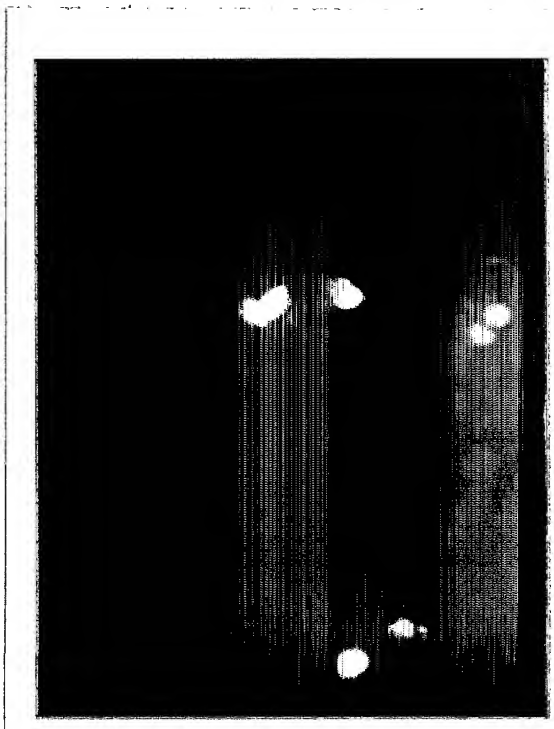

FIG. 1B

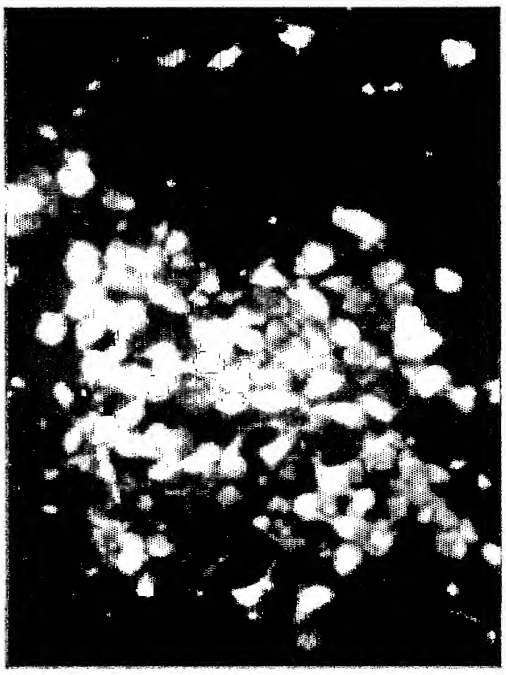

FIG. 1D

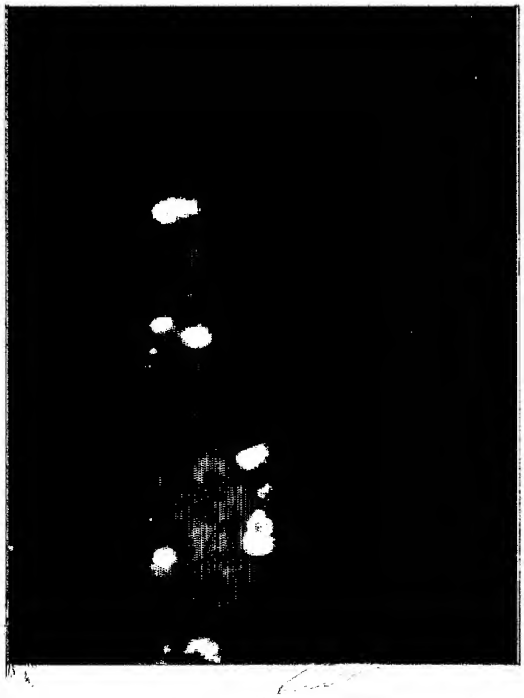

FIG. 1A

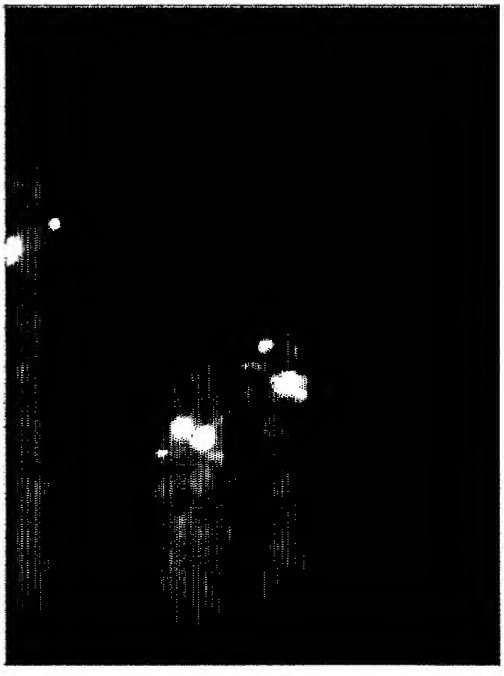

FIG. 1C

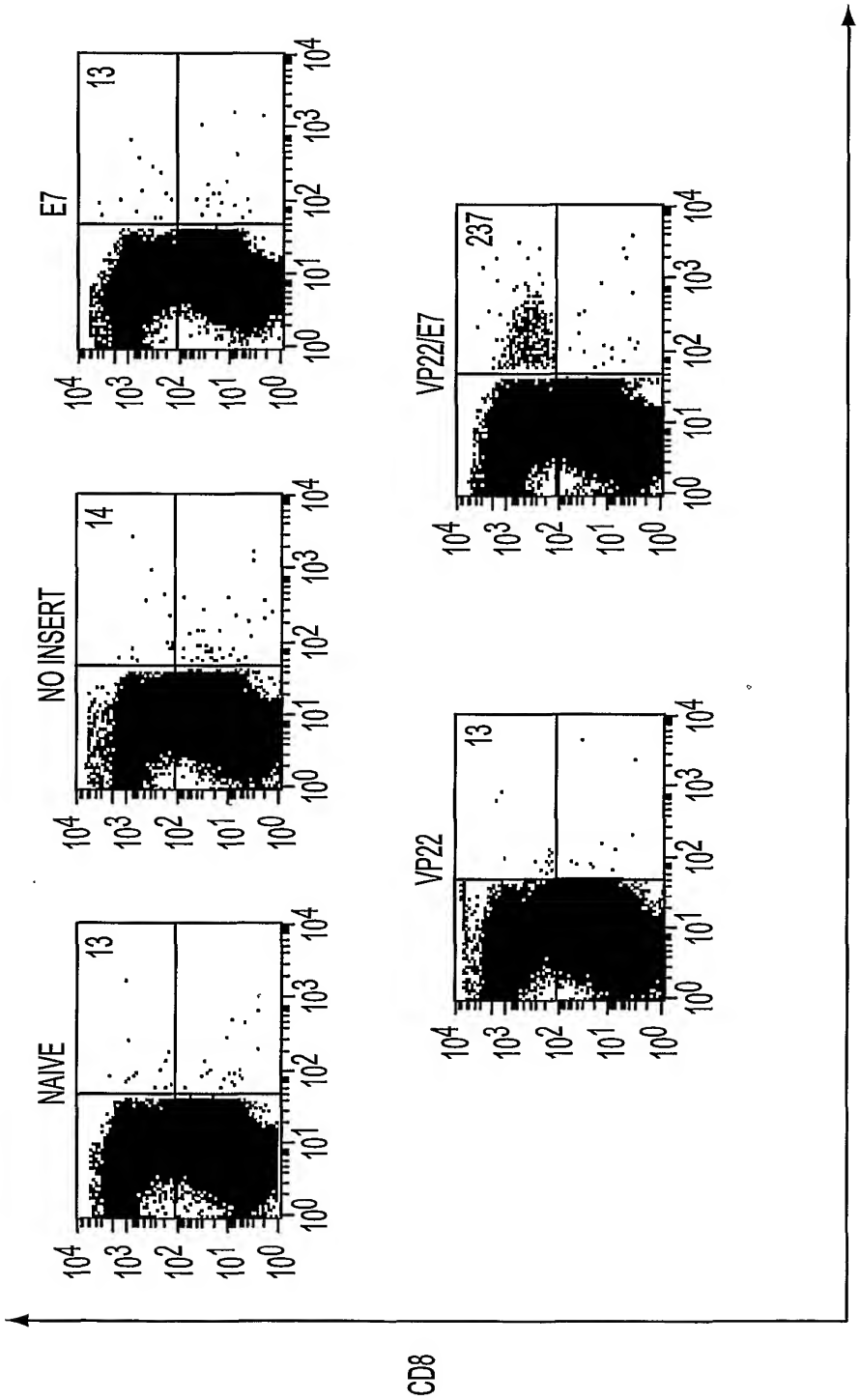

FIG. 2A

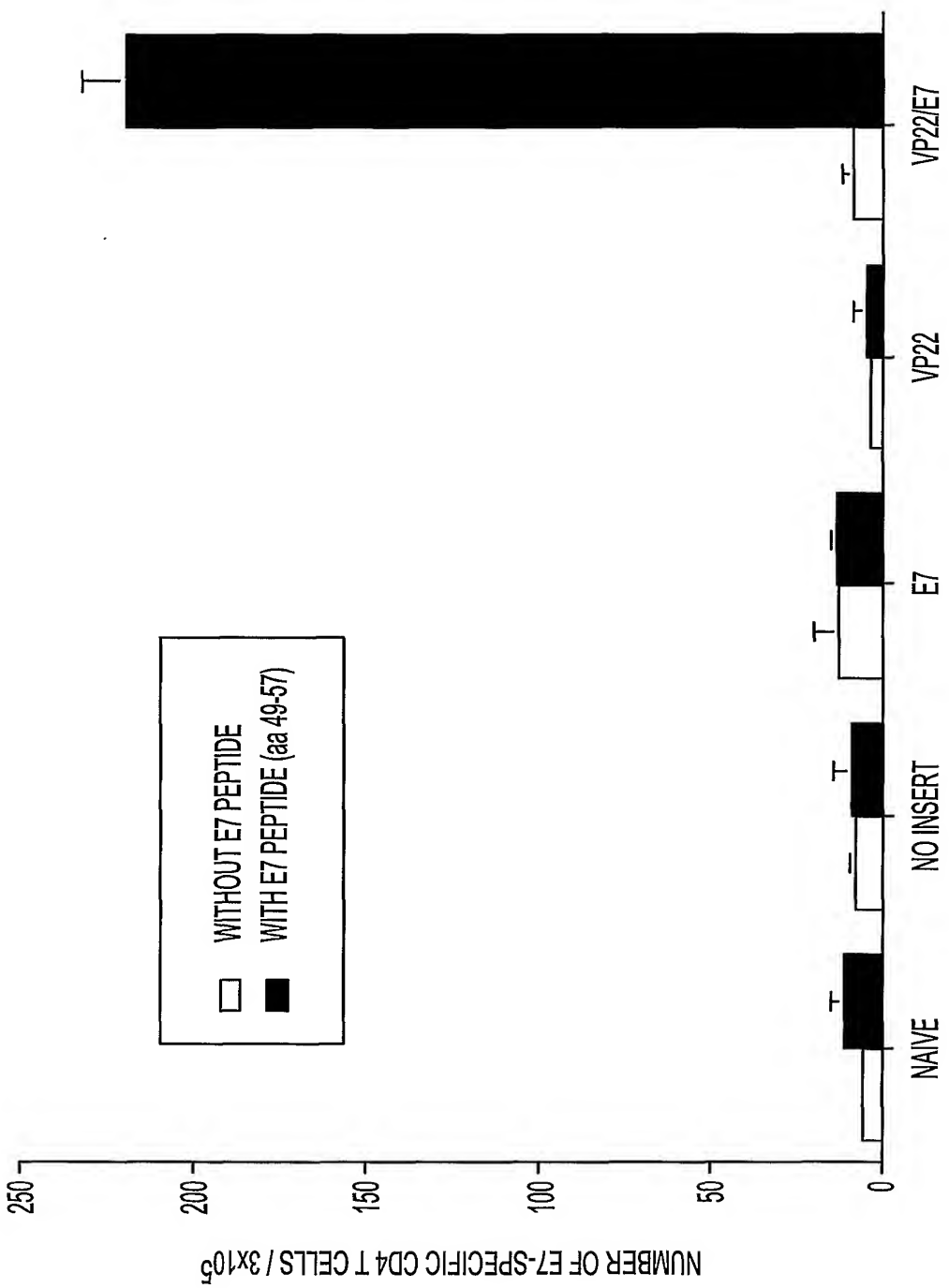
NUMBER OF E7-SPECIFIC CD4 T CELLS / 3x10^5
250
200
150
100
50
0
WITHOUT E7 PEPTIDE
WITH E7 PEPTIDE (aa 49-57)
NAIVE
NO INSERT
E7
VP22
VP22/E7

FIG. 2B

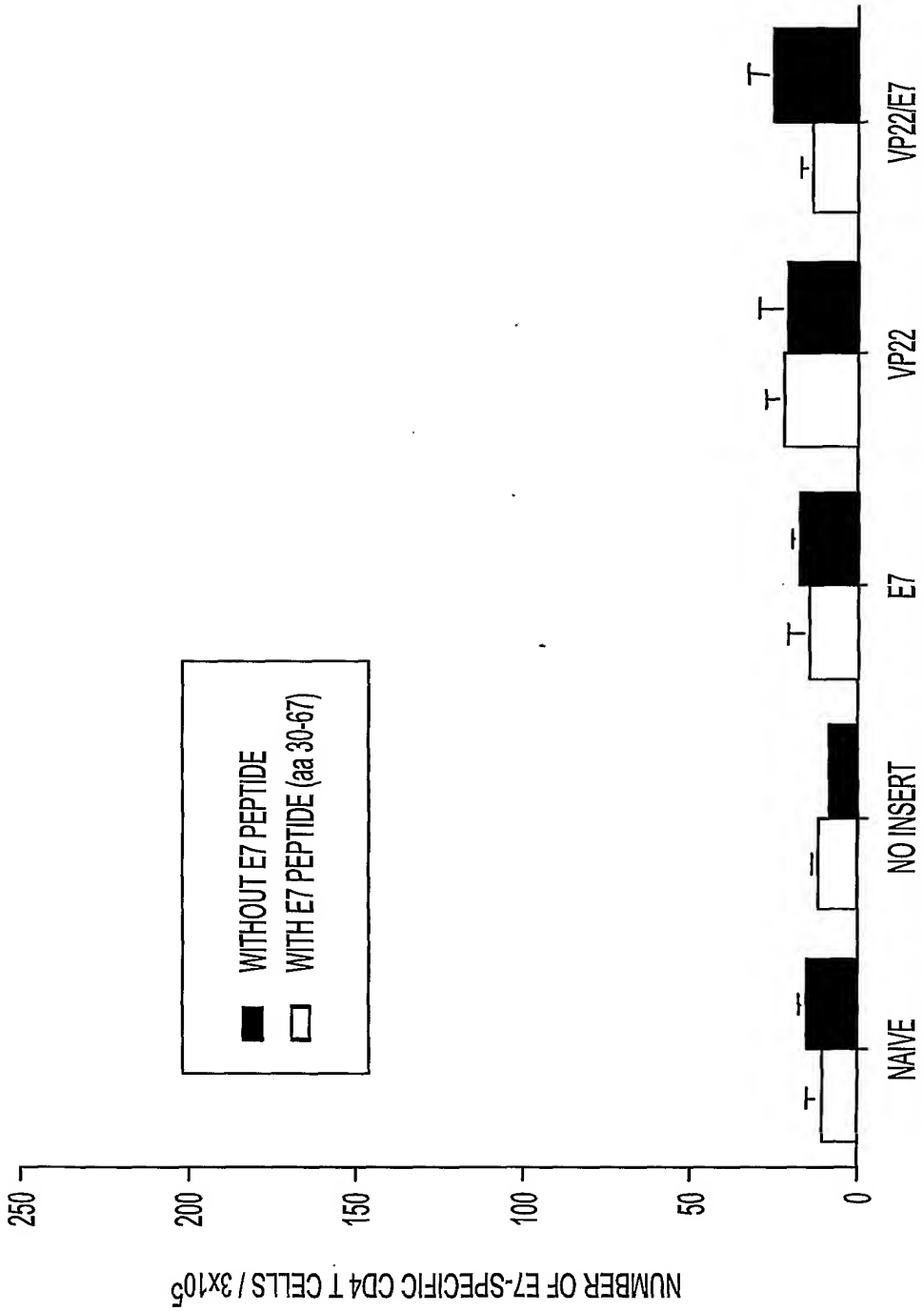
NUMBER OF E7-SPECIFIC CD4 T CELLS / 3x10^5
250
200
150
100
50
0
WITHOUT E7 PEPTIDE
WITH E7 PEPTIDE (aa 30-67)
NAIVE
NO INSERT
E7
VP22
VP22/E7

FIG. 2C

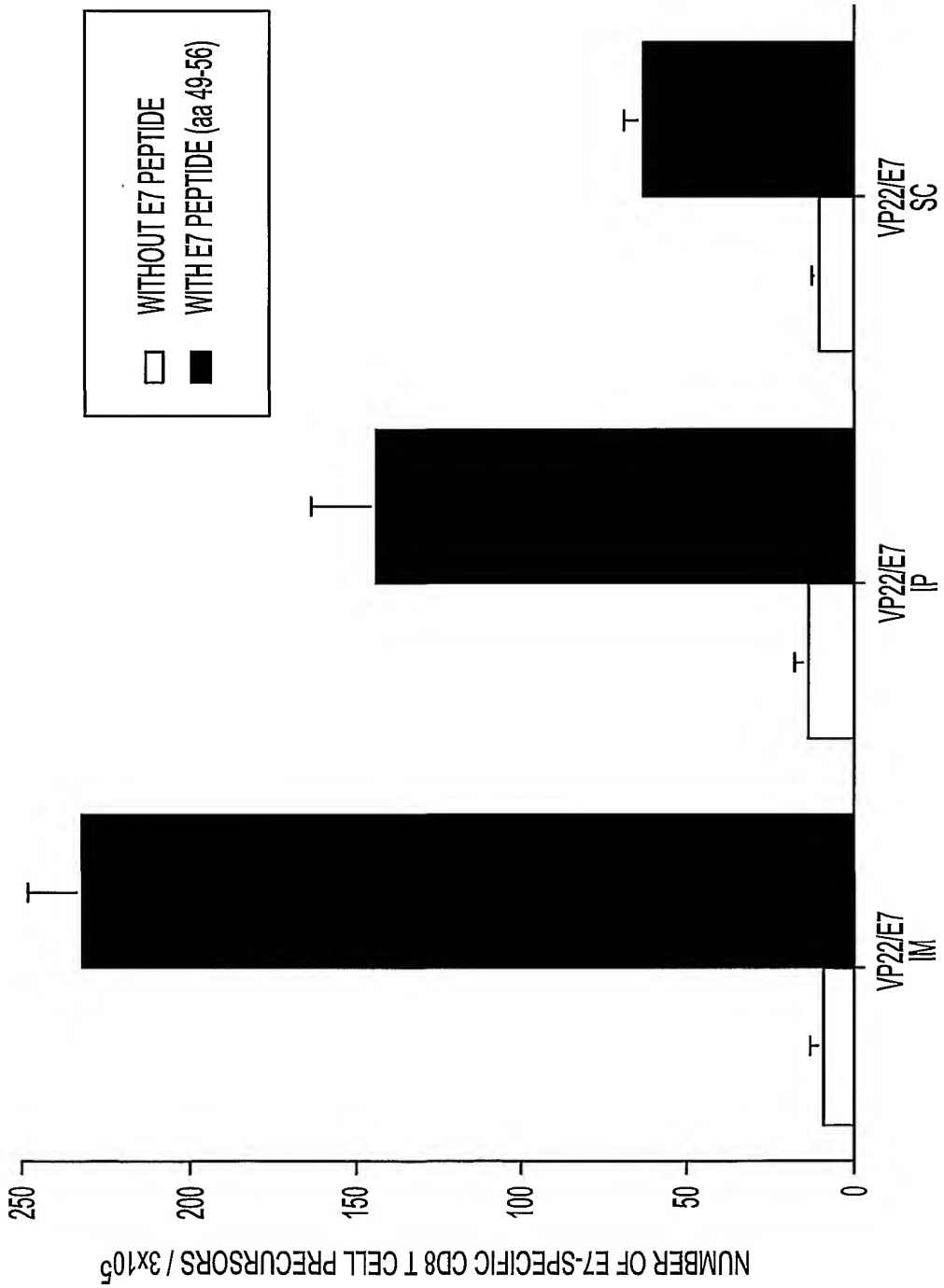
WITHOUT E7 PEPTIDE
WITH E7 PEPTIDE (aa 49-56)
NUMBER OF E7-SPECIFIC CD8 T CELL PRECURSORS / 3x10^5
250
200
150
100
50
0
VP22/E7 IM
VP22/E7 IP
VP22/E7 SC

FIG. 3A

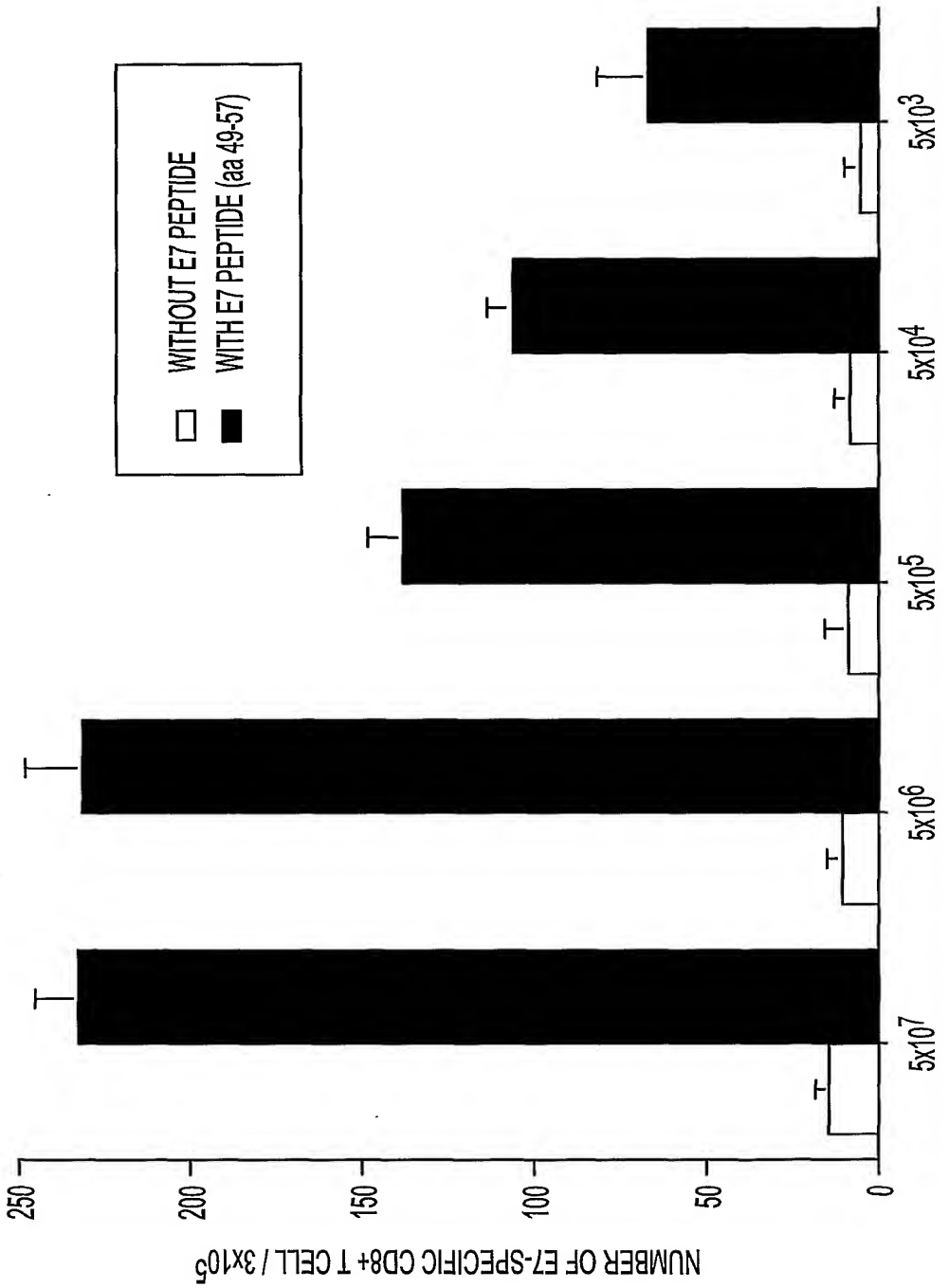
WITHOUT E7 PEPTIDE
WITH E7 PEPTIDE (aa 49-57)
NUMBER OF E7-SPECIFIC CD8+ T CELL / 3x10^5
250
200
150
100
50
0
5x10^7
5x10^6
5x10^5
5x10^4
5x10^3

FIG. 3B

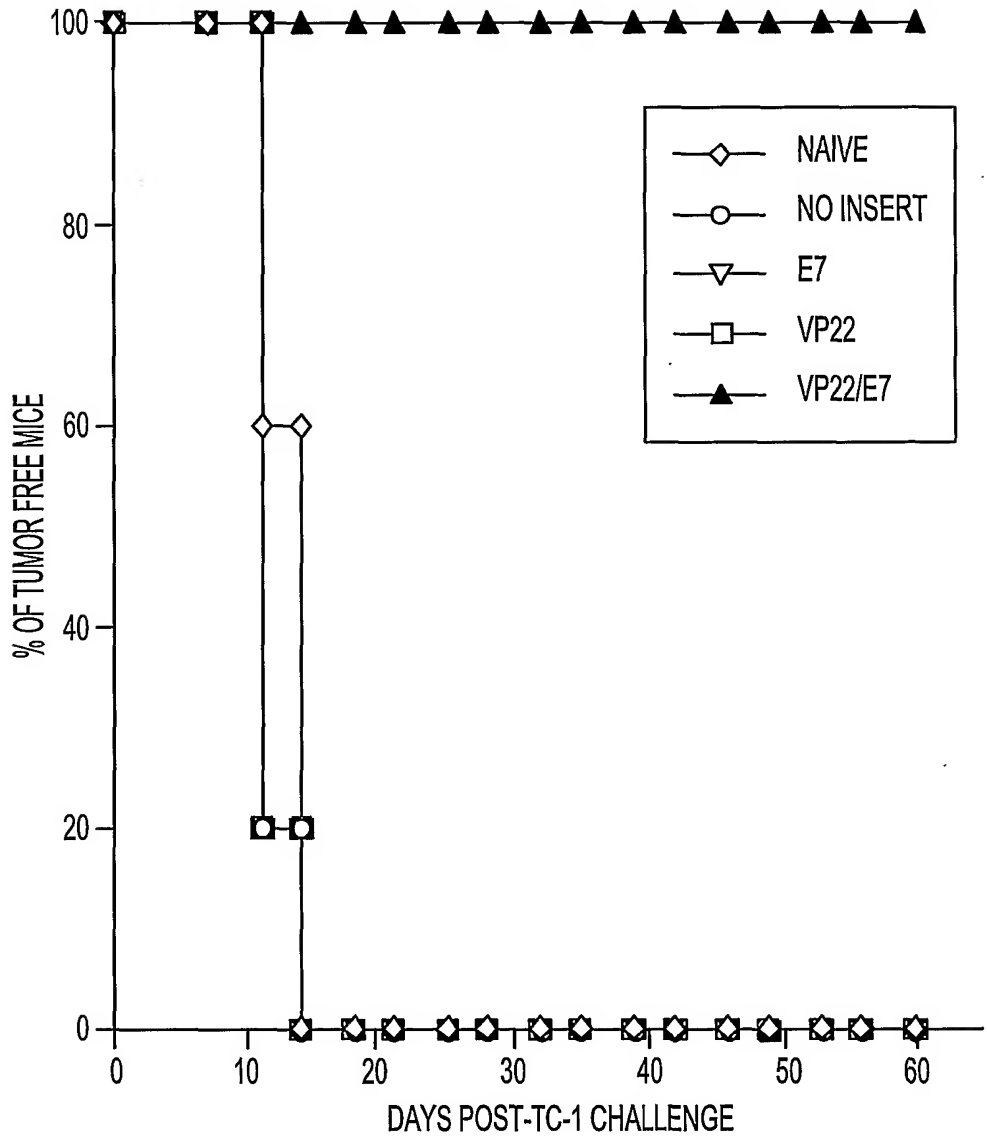
100
80
60
40
20
0
% OF TUMOR FREE MICE
0
10
20
30
40
50
60
DAYS POST-TC-1 CHALLENGE
NAIVE
NO INSERT
E7
VP22
VP22/E7

FIG. 4

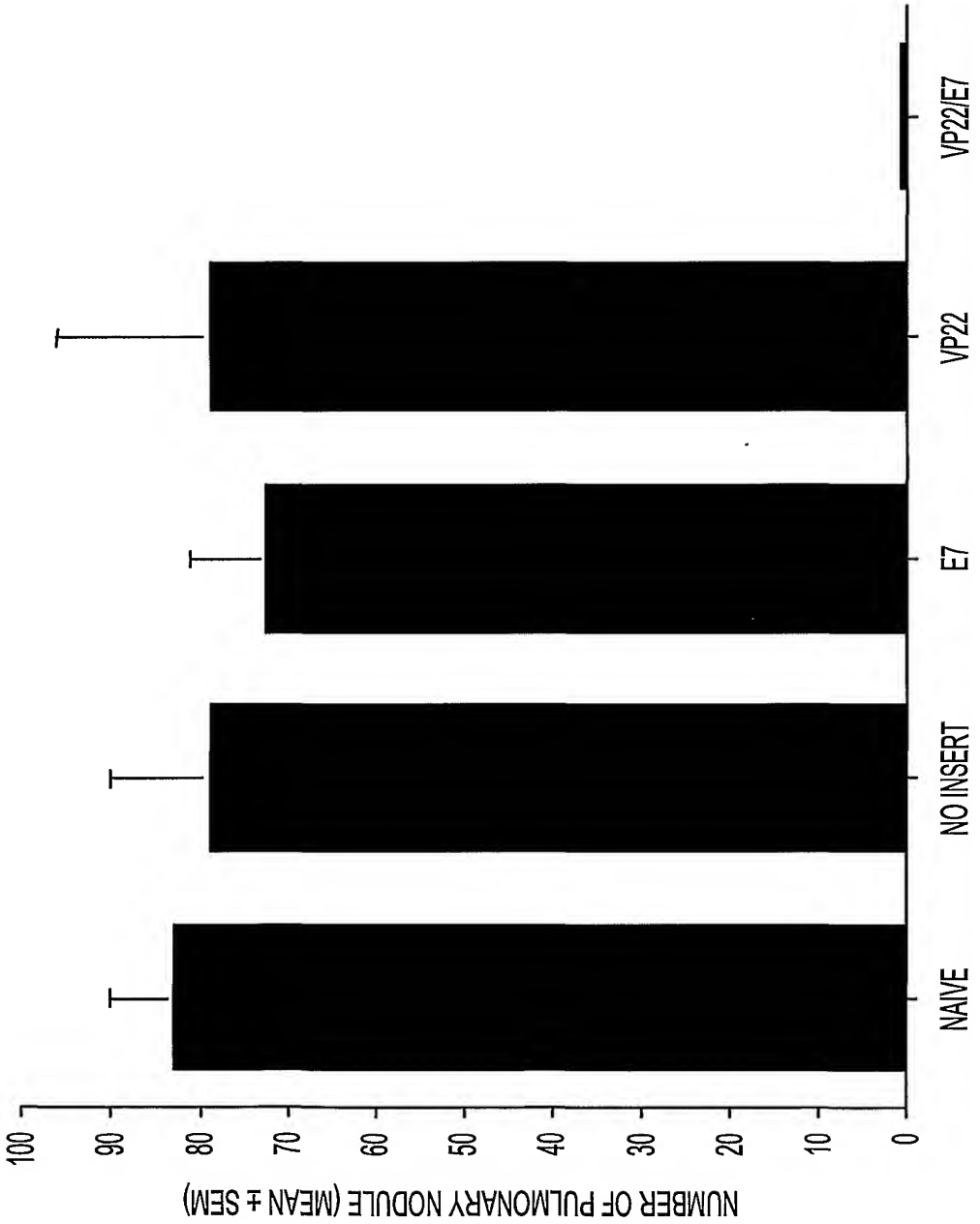
NUMBER OF PULMONARY NODULE (MEAN ± SEM)
0
10
20
30
40
50
60
70
80
90
100
NAIVE
NO INSERT
E7
VP22
VP22/E7

FIG. 5A

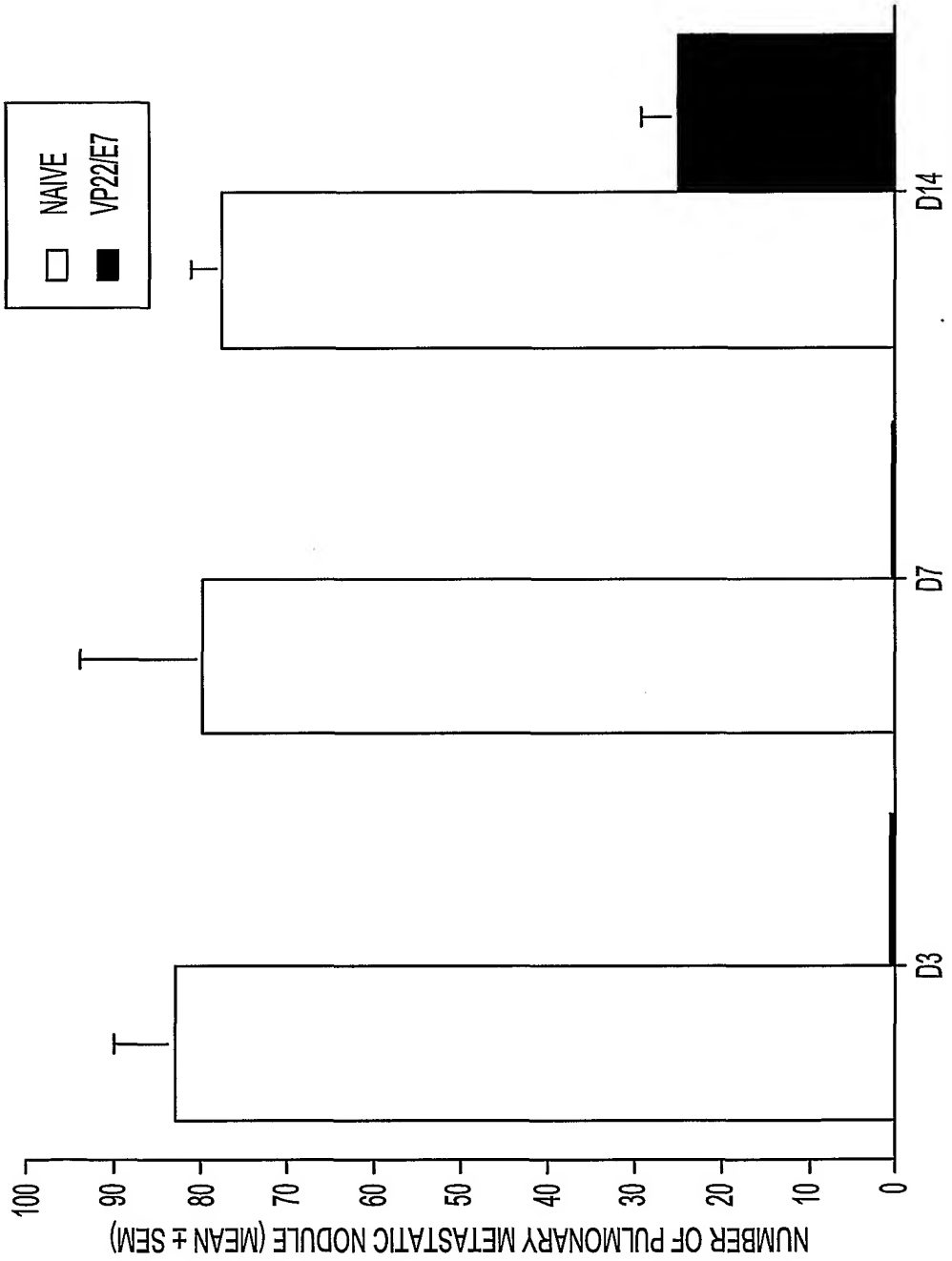
NAIVE
VP22/E7
NUMBER OF PULMONARY METASTATIC NODULE (MEAN ± SEM)
0
10
20
30
40
50
60
70
80
90
100
D3
D7
D14

FIG. 5B

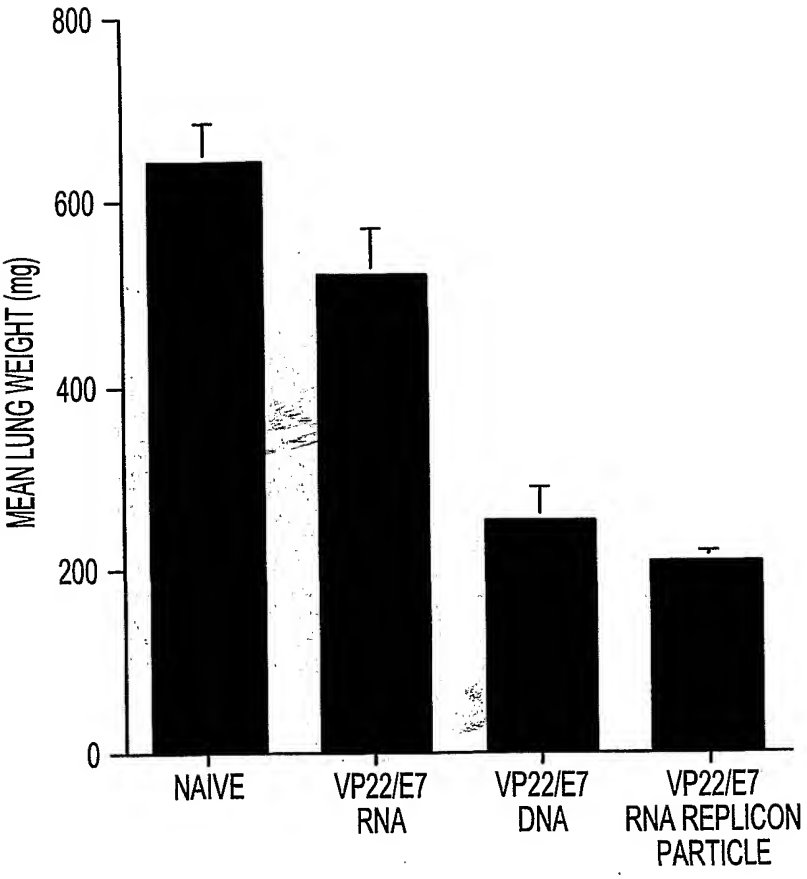
800
600
400
200
0
MEAN LUNG WEIGHT (mg)
NAIVE
VP22/E7 RNA
VP22/E7 DNA
VP22/E7 RNA REPLICON PARTICLE

FIG. 6A

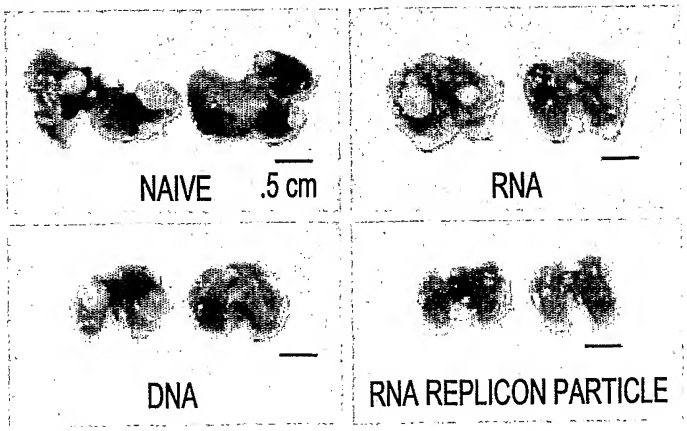
NAIVE
.5 cm
RNA
DNA
RNA REPLICON PARTICLE

FIG. 6B

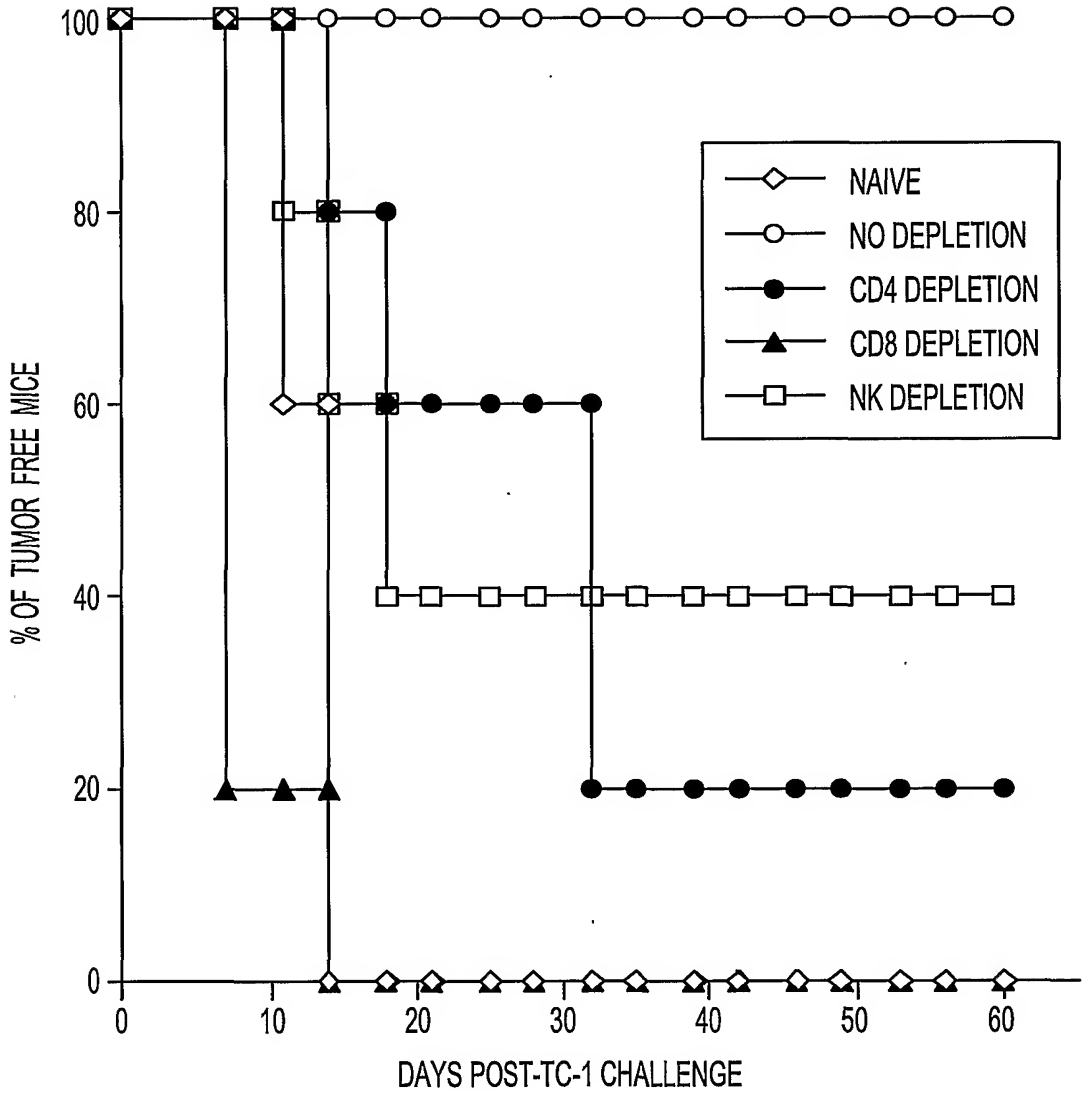
100
80
60
40
20
0
% OF TUMOR FREE MICE
0
10
20
30
40
50
60
DAYS POST-TC-1 CHALLENGE
NAIVE
NO DEPLETION
CD4 DEPLETION
CD8 DEPLETION
NK DEPLETION

FIG. 7

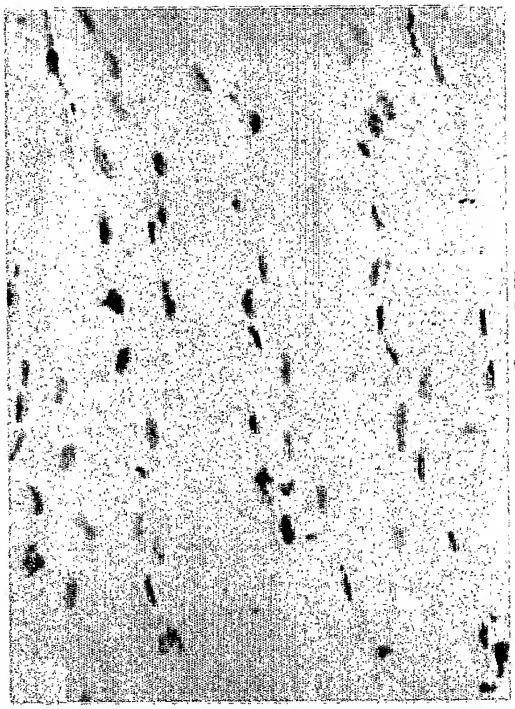

FIG. 8B

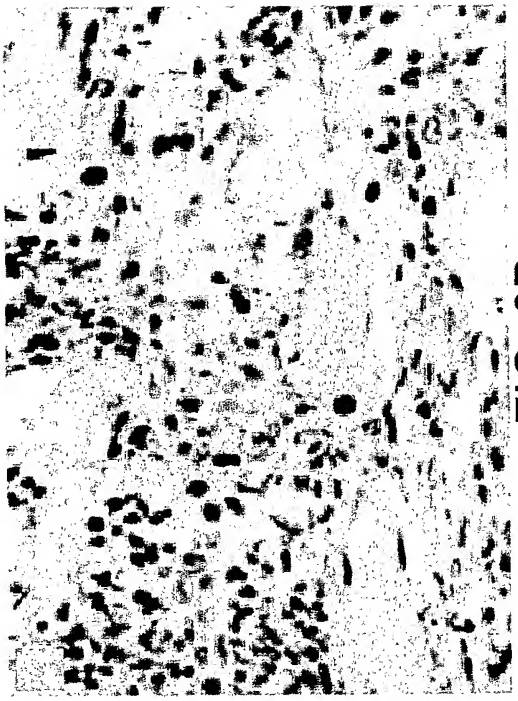

FIG. 8D

FIG. 8A

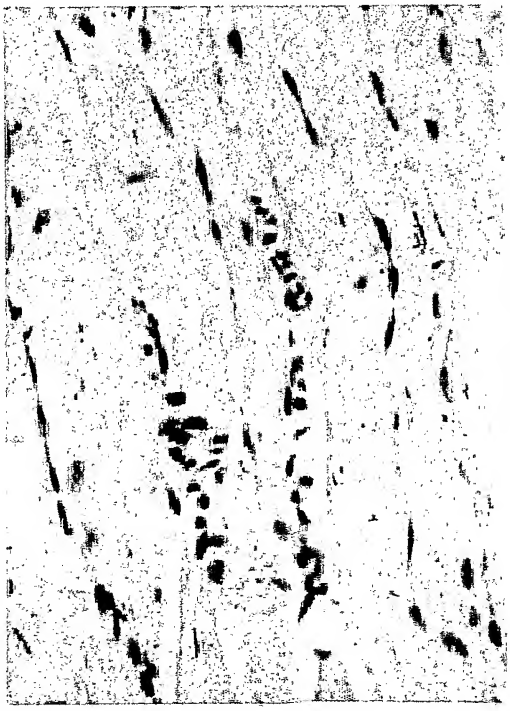

FIG. 8C

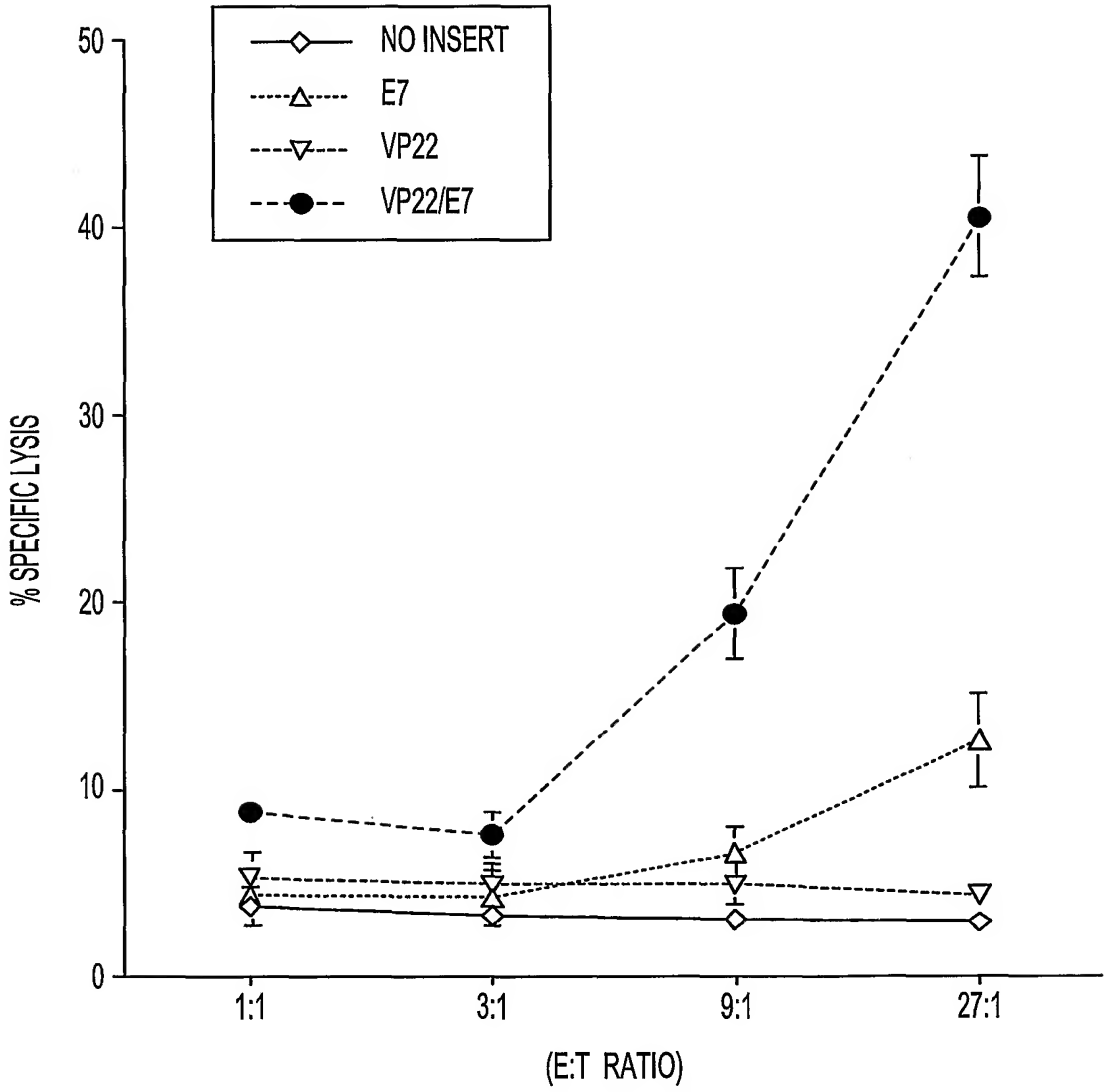
NO INSERT
E7
VP22
VP22/E7
% SPECIFIC LYSIS
50
40
30
20
10
0
1:1
3:1
9:1
27:1
(E:T RATIO)

FIG. 9